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Linking bacterial communities on grapes to biogenic amines production in musts

Rita Isabel da Silva Calisto

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Rita Isabel da Silva Calisto

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto

Mestrado em Biologia Celular e Molecular

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Esta dissertação foi realizada no âmbito do **Projecto PP-IJUP2014-SOGRAPE-06,** dos Projetos Pluridisciplinares IJUP-Empresas.

Charles Darwin (1837)

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Resumo

A indústria vinícola é um negócio não só com um grande valor histórico, mas também com particular importância na economia portuguesa. A produção de vinho está dependente de comunidades microbianas presentes, não só no material enológico, como também da matéria prima, ou seja, nas uvas. Apesar da comunidade bacteriana responsável pela fermentação estar bem descrita, a comunidade associada às uvas ainda não se encontra devidamente caracterizada. Durante os processos de vinificação, para além dos processos essenciais para a produção de vinho, como a fermentação, a comunidade bacteriana pode também produzir moléculas indesejáveis, que podem ser nefastas para o vinho e para a saúde humana, como as Aminas Biogénicas (ABs). Estas moléculas são normalmente formadas pela descarboxilação de aminoácidos por enzimas especificas.

Mostos da região Alentejana demonstraram conter níveis mais elevados de ABs sendo a sua origem desconhecida. No entanto, este problema não tem sido verificado em mostos produzidos na região do Douro Superior. Deste modo, o objetivo desta dissertação foi isolar e analisar a comunidade bacteriana presente nas uvas destas duas regiões vinícolas portuguesas, e estudar a sua capacidade para a produção de ABs.

Pelo isolamento em, obtivemos 168 isolados bacterianos das uvas, sendo 14 isolados do Douro e 154 do Alentejo. As uvas provenientes do Alentejo revelaram ter uma comunidade bacteriana maior e mais diversa em relação às uvas do Douro. Até ao momento, 80 isolados foram identificados com base no gene 16S rRNA, sendo o phylum predominante *Firmicutes* (56 %), tendo também sido encontrados os phyla: *Actinobacteria* (27%), *Alphaproteobacteria* (14%), *Gammaproteobacteria* (3%) and *Bacteroidete* (1%). Assim, o estudo por técnicas dependentes de cultura aponta para uma forte influência da biogeografia na comunidade bacteriana das uvas. No entanto, a nossa abordagem por um método independente de cultura, por Eletroforese em Gel com Gradiente Desnaturante, não revelou estas diferenças, mas, pelo contrário, demonstrou similaridade entre as populações das uvas e de mosto das duas regiões

O estudo do potencial bioativo das bactérias para a produção de metabolitos secundários, revelou uma prevalência de genes bioativos em grande potencial das bactérias provenientes do Alentejo.

A comunidade bacteriana do Alentejo revelou também um grande potencial para a produção de ABs, especialmente para a produção de putrescina, já que um número maior de bactérias demonstrou a presença de genes codificantes de enzimas responsáveis pela produção desta molécula (32 isolados de 97 isolados estudados).

Paralelamente, os níveis de ABs foram quantificados por LC-MS, tendo-se confirmado dados anteriores, sendo que os níveis de aminas nas uvas e nos mostos foram bastante mais elevados no Alentejo, comparativamente ao Douro (diferença de 11 vezes).

O número mais elevado de bactérias bem como a maior diversidade associado a um maior potencial para a produção de ABs poderão explicar os níveis elevados de Aminas Biogénicas nos mostos alentejanos. Além disso, a análise da superfície das uvas provenientes do Alentejo, microscopia eletrónica de varrimento, revelou um bio filme microbiano distribuído desigualmente, frequentemente associado a material mucilaginoso. Este material pode ser uma fonte nutricional para as bactérias produtoras de ABs presentes na superfície da uva, justificando assim o rápido aumento nos níveis de putrescina das uvas maduras para os primeiros mostos no Alentejo.

Palavras-Chave: Uva, bactérias, diversidade, aminas biogénicas, DGGE, SEM, potencial bioativo, PKS-I, NRPS, *agdi*, tyrd, *odc*, *ldc*, putrescina, Douro, Alentejo, LC-MS

Abstract

The wine industry is an exceptionally ancient business as well as a particularly important in Portuguese economy. Turning grape juice into wine requires and depends on microbial communities that can be present in wine equipment but also has origin in the grapes. Although strains responsible for wine fermentation are well known and studied, the grape associated bacterial communities are still poorly characterized. Along with the essential wine reactions that are mainly dependent on microbial communities, bacteria can also produce molecules that can be prejudicial to wine and to human health, like Biogenic Amines (BAs). These are usually formed through the decarboxylation of amino acids by specific enzymes.

Red musts in the Alentejo region demonstrated to have high levels of BAs which origin is unknown. However, this problem is not present in musts produced by the same company in the Douro Superior region. Thus, the aim of this dissertation was to isolate and analyse the bacterial community present in grapes from these two wine producing Portuguese regions and to analyse the capacity of the bacterial isolates for the production of BAs.

Bacterial isolation allowed the obtainment of 168 isolates, 14 from Douro and 154 from Alentejo. Alentejo grapes revealed to have a higher and more diverse bacterial community than Douro grapes. Until now, 80 isolates were identified based on the 16S rRNA gene being *Firmicutes* the dominant group (56%), but *Actinobacteria* (27%), *Alphaproteobacteria* (14%), *Gammaproteobacteria* (3%) and *Bacteroidete* (1%) were also found. Our culture-dependent study points out to a strong influence of biogeography on the bacterial community of the grapes. However, the culture-independent approach by Denaturing Gradient Gel Electrophoresis (DGGE) did not reveal such differences, on the contrary the populations from the grapes and musts of the two regions were similar.

The search of bioactive potential for the production of secondary metabolites in the bacteria isolated, revealed a higher prevalence of bioactive genes among bacteria in Alentejo.

Bacteria from Alentejo revealed a much higher potential for the production of BAs, especially for putrescine, because a higher number of bacteria possessed the genes for the enzymes responsible for this molecule (32 isolates out of the 97 studied).

In parallel, chemical analysis of the levels of BAs in grapes and musts confirmed previous data that demonstrate much higher levels in Alentejo comparatively to Douro (a 11-fold difference).

Higher bacterial number and diversity associated to a high BAs potential production in Alentejo comparatively to Douro may explain the high levels of BAs in Alentejo musts. Furthermore, the analysis of grape surfaces in Alentejo revealed an uneven microbial biofilm often associated to mucilaginous-like material. This could be the nutritional source for BAs producing bacteria justifying the relevant increase in putrescine levels between the mature grapes and the freshly produced musts in Alentejo.

Keywords: Grape, bacteria, diversity, biogenic amines, DGGE, SEM, bioactive potential, PKS-I, NRPS, *agdi*, tyrd, *odc*, *ldc*, putrescine, Douro, Alentejo, LC-MS

Table of contents

Table Index

Figure Index

Abbreviations

%	Percent sign
®	registered trademark
^{0} C	Degree Celsius
AAB	Acetic Acid Bacteria
ABs	Aminas Biogénicas
AGDI	Agmatine deiaminase
BAs	Biogenic Amines
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CFU	Colony forming unit
DAO	Diamine oxidase
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
e.g	*Exempli gratia*
EDTA	Ethylenediamine tetraacetic acid
g	Gram
HDC	Histidine decarboxylase
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography – Mass Spectrometry
LDC	Lysine decarboxylase
M	Molar
MAO	Monoamine oxidase
mg	Milligram
mg/L	Milligram per Litre
min	Minutes
mL	Milliliter
ML	Maximum Likewood
mM	MilliMolar
MRS - A	Man, Rogosa and Sharpe Agar medium
NA	Nutrient Agar medium
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
nMDS	Non-Metric Multidimensional Analysis Scaling
NRPS	Nonribosomal peptide synthetases

ODC	Ornithine decarboxylase
OM	Optical microscopy
PCR	Polymerase Chain Reaction
pH	Potential of Hidrogen
PKS	Polyketide synthases
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Seconds
SEM	Scanning Electron Microscopy
***sp*.**	Specie
spp.	Specie
SRM	Simple Race Mode
TAE	Tris-Acetate EDTA
TDC	Tyrosine decarboxylase
™	Trademark symbol
UV	Ultraviolet
VBNC	Viable but nonculturable
µL	Microliter
µm	Micrometer
µM	Micromolar

1. Introduction

1.1. Wine and Microorganisms

The importance of wine in human societies remounts centuries ago, being nowadays, recognized for its health benefits and social and economic relevance. The earliest evidence of wine goes back to, approximately, 7000 years ago and its production as evolved with the Humanity itself and played very important roles in many civilizations, such as, the disinfection of water, the development of pottery and of trade routes (one of the most significant commercial products) (Jones *et al.*, 2005, Charters, 2006, Estreicher, 2013). With the advances of the human society and development of knowledge through the centuries, the wine making process as evolved from a simple pot with macerated grapes to a meticulous and calculated technology (Estreicher, 2013).

The production of wine is based on chemical modifications that occur in grape juice. The transformation of juice into wine is mainly induced by microorganisms able to ferment the musts as a consequence of their metabolic activity (Renouf *et al.*, 2005). These biochemical processes are mainly due to yeasts, essentials for alcoholic fermentation, and bacteria (Lactic Acid Bacteria), that carry out the malolactic fermentation.

Since the first scientific studies on wine microbiology, by Pasteur (1872), it has been confirmed, that grape surfaces are colonized by many microorganisms. The majority of wine related studies are focused on the microorganisms with oenological interest such as *Saccharomyces cerevisiae*, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB), microorganisms involved in alcoholic and malolactic fermentations (Renouf *et al.*, 2005, Ancín-Azpilicueta *et al.*, 2008). Furthermore, many studies have been performed in the winery environment, for better understanding of the role of microorganisms in wine' sanitary conditions and, therefore, wine spoilage (Garijo *et al.*, 2008, Marques *et al.*, 2008, Del Prete *et al.*, 2009). Even though the microbiome present on grapes plays a main role in the health of vine plants, in the ripening of the fruit (Martins *et al.*, 2013) and in the wine production, there is still a huge gap of knowledge on the grape's epiphytic bacteria (Renouf *et al.*, 2005, Estreicher, 2013). Also, the data about the microbiome associated with grapevine plants and its biogeography is still scarce (Coombe, 1992, Arena & Manca de Nadra, 2001, Prakitchaiwattana *et al.*, 2004, Renouf *et al.*, 2005, Renouf *et al.*, 2007, Bokulich *et al.*, 2014, Gilbert *et al.*, 2014, Pinto *et al.*, 2014).

In grapes, bacteria, especially specific groups, like Lactic acid bacteria (approximately 10^2 CFU/g) and Acetic acid bacteria (less than 10 CFU/g), are typically in

much lower numbers than yeasts and are, thus, hard to detect, which contributes for the lack of information regarding grape's bacterial communities. Their isolation implies specific isolation and culture procedures (Renouf *et al.*, 2005, Renouf *et al.*, 2007, Francesca *et al.*, 2011, Barata *et al.*, 2012).

Recently some studies have been done on the diversity and dynamics of epiphytic microbiota of grapes. Pinto and collaborators (2014) have described, in a temporal dynamic study, the microbial genera and species present in grape vines. Using grapes from a Portuguese wine region, Bairrada, they showed that the prokaryotic community present was, mainly, constituted by members of the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria*. The most abundant classes were *Bacilli*, *Alphaproteobacteria*, *Negativicutes*, *Betaproteobacteria* and *Gammaproteobacteria*. Non-classified organisms were also present. The main families were *Streptococcaceae*, *Enterobacteriaceae*, *Pseudomonadaceae* and Moraxellaceae followed by Leuconostocaceae, *Comamonadaceae*, *Veillonellaceae*, *Xanthomonadaceae*, *Sphingomonadaceae* and *Neisseriaceae*. Lactic acid bacteria from the families *Carnobacteriaceae*, *Enterococcaceae*, *Leuconostocaceae* and *Streptococcaceae* were detected. Furthermore, this study corroborated other scientific studies as it demonstrated that the microbial community changed during the vegetative cycle of the vine plant. Curiously, this and other biodiversity studies on microbial communities of grapes have not detected the main bacteria present in wine, such as *Oenococcus oeni*. The majority of the species described are not related to wine production (Bae *et al.*, 2006, Nisiotou *et al.*, 2011). Furthermore, Barata *et al.* (2012) and Renouf *et al.* (2005, 2007) were only able to recover *O. oeni* from grapes when using a specific enrichment cultures methodology suitable for elicit minority populations. Some LAB isolated from grapes belonged to the genera *Lactobacillus*, *Enterococcus*, *Weisella*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, but not all of these genera were isolated in all the studies. Regarding AAB, bacteria commonly more present on rotten grapes (Barbe *et al.*, 2001), the genera *Gluconobacter* and *Acetobacter* are the more detected (Barata *et al.*, 2012). Other studies (Gilbert *et al.*, 2014, Zarraonaindia *et al.*, 2015) focused on the differences of bacterial population within the vine plant, analysing, separately, the bacteria present in leafs, grapes, flowers and soil. Once more, *Proteobacteria* was the grapes' most dominant phylum (*Pseudomonas*, *Achromobacter*, *Massilva*, *Cellvibrio*). In grapes, *Firmicutes*, *Acidobacteria* and *Bacteroidetes* were also detected. Leaves, flowers, bark and soil were described as being colonized mainly by *Proteobacteria* (*Xanthobacter*, *Sphingomonas*, *Methylobacterium*, *Rhizobium*, among others), *Actinobacteria* (*Micrococcus*, *Cellulomonas* and others) and *Firmicutes* (*Streptococcus*, *Bacillus*, *Paenibacillus* and *Clostridium*) (Gilbert *et al.*, 2014).

A more complete list of previously described grape's epiphytic bacteria can be found in Table 1.

Table 1 – List of bacteria described as isolated from grape or grape related sites (soil, leafs, bark, flowers). Modified from Barata *et al*., 2012 and Martins *et al*., 2013 with references there in.

Genus	Species	Phylum/Class
Acetobacter	*Acetobacter aceti, A. pasteurianus, A. cerevisiae, A. orleanensis, A. syzygii*	*Proteobacteria/ Alphaproteobacteria*
Achromobacter	*Achromobacter sp.*	*Proteobacteria/ Betaproteobacteria*
Acinetobacter	*Acinetobacter spp.*	*Proteobacteria/ Gammaproteobacteria*
Agrobacterium	*Agrobacterium tumefaciens*	*Proteobacteria/ Alphaproteobacteria*
Bacillus	*Bacillus mycoides, B. subtilis, B. pumilus, Bacillus. sp.*	*Firmicutes*
Brevibacterium	*Brevibacterium sp.*	*Actinobacteria*
Burkholderia	*Burkholderia vietnamiensis*	*Proteobacteria/ Betaproteobacteria*
Cellulomonas	*Cellulomonas sp.*	*Actinobacteria*
Cellvibrio	*Cellvibrio sp.*	*Proteobacteria/ Gammaproteobacteria*
Citrobacter	*Citrobacter freundii*	*Proteobacteria/ Gammaproteobacteria*
Curtobacterium	*Curtobacterium spp.*	*Actinobacteria*
Enterobacter	*E. gergoviae, E.ludwigii, E. spp, Enterobacter. sp.*	*Proteobacteria/ Gammaproteobacteria*
Enterococus	*Enterococus durans, E. faecium, E. avium, E. hermaniensis, E. durans*	*Firmicutes*

Erwinia	*Erwinia spp.*	*Firmicutes*
Gluconoacetobacter	*Gluconoacetobacter hansenii, Gl. saccharivorans, Gl. intermedius*	*Proteobacteria/ Alphaproteobacteria*
Gluconobacter	*Gluconobacter oxydans, G. cerinus*	*Proteobacteria/ Alphaproteobacteria*
Klebsiella	*Klebsiella oxytoca*	*Proteobacteria/ Gammaproteobacteria*
Kocuria	*Kocuria kristinae*	*Actinobacteria*
Lactobacillus	*Lactobacillus plantarum, L. hilgardii, L. casei, L. sanfranciscansis L. lindneri, L. kunkeei L. brevis, L. kefiri, L. mali ,*	*Firmicutes*
Lactococcus	*Lactococcus lactis*	*Firmicutes*
Leifsonia	*Leifsonia xyli*	*Actinobacteria*
Leuconostoc	*Leuconostoc fallax, Lc. mesenteroides*	*Firmicutes*
Massilia	*Massilia sp.*	*Proteobacteria/ Betaproteobacteria*
Methylobacterium	*Methylobacterium sp.*	*Proteobacteria/ Alphaproteobacteria*
Micrococcus	*Micrococcus sp.*	*Actinobacteria*
Oenococcus	*Oenococcus oeni*	*Firmicutes*
Paenibacillus	*Paenibacillus sp*	*Firmicutes*
Pantoea	*P. dispersa, Pantoea sp.*	*Proteobacteria/ Gammaproteobacteria*
Pediococcus	*Pediococcus parvulus, P. damnosus, P. acidilactici*	*Firmicutes*

Providencia	*Providencia rettgeri*	*Proteobacteria/ Gammaproteobacteria*
Pseudomonas	*P. jessenii, Pseudomonas sp.*	*Proteobacteria/ Gammaproteobacteria*
Rhizobium	*Rhizobium sp.*	*Proteobacteria/ Alphaproteobacteria*
Serratia	*S. rubidae, S. marcescens, Serratia spp*	*Proteobacteria/ Gammaproteobacteria*
Sphingomonas	*Sphingomonas sp.*	*Proteobacteria/ Alphaproteobacteria*
Staphylococcus	*S. saprophyticus, Staphylococcus spp.*	*Firmicutes*
Stenotrophomonas	*Stenotrophomonas maltophila*	*Proteobacteria/ Gammaproteobacteria*
Streptococcus	*Streptococcus sp.*	*Firmicutes*
Streptomyces	*Streptomyces sp.*	*Actinobacteria*
Tatumella	*Tatumella ptyseos*	*Proteobacteria/ Gammaproteobacteria*
Weissella	*W. paramesenteroides, Weissella sp.*	*Firmicutes*
Xanthobacter	*Xanthobacter sp.*	*Proteobacteria/ Alphaproteobacteria*
Xanthomonas	*Xanthomonas sp.*	*Proteobacteria/ Alphaproteobacteria*

Despite the fundamental roles in wine production, microorganisms may also be prejudicial with the production of undesired molecules, such as biogenic amines (BAs).

1.2. Biogenic Amines – overview

Biogenic amines are nitrogenous compounds normally produced as a result of normal metabolism of plants, animals and microorganisms (Karovičová, 2003, Ancín-Azpilicueta *et al.*, 2008). Their chemical structure can be classified as aliphatic (cadaverine, spermine, putrescine), aromatic (tyramine, phenylethylamine) and heterocyclic (histamine, tryptamine) (Karovičová, 2003, Anlı & Bayram, 2008) (Fig.1). BAs are precursors of several compounds essential in physiological mechanisms such as hormone synthesis and cell proliferation (Lonvaud-Funel, 2001, Marques *et al.*, 2008). However, in high amounts, BAs can, potentiated by ethanol, cause toxic effects as vomiting and hypertension, especially, in sensitive humans (Anlı & Bayram, 2008, Spano *et al.*, 2010, Ladero *et al.*, 2012), and affect organoleptic features of wine (Smit, 2008), being, thus, imperative for the wine industry to control BAs levels (Marques *et al.*, 2008). Additionally, some E.U. countries are imposing limits in BAs levels in wine (*e.g.* in Germany, the maximum histamine allowed is 2 mg/L), influencing commercial transactions between countries (Inês *et al.*, 2009).

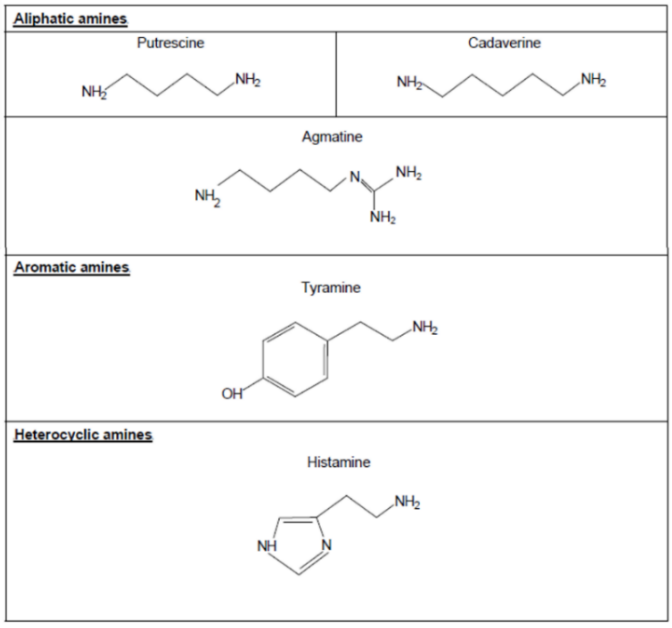


Figure 1 – Chemical structure of some Biogenic Amines with oenological importance - Putrescine, Cadaverine, Agmatine, Tyramine and Histamine (modified from Smit, 2008)

The presence of BAs in wine is not uncommon as bacteria can produce these molecules by decarboxylation or deamination of amino acids, using substrate-specific enzymes (Torrea & Ancín, 2002). The production of BAs is dependent on three factors: (1) availability of amino acid precursors, (2) presence of decarboxylase-positive organisms, and (3) conditions for the growth of bacteria and for decarboxylase synthesis and activity. The main BAs found in wine are putrescine, histamine, tyramine, and

cadaverine, formed through different pathways. Putrescine can be produced by two enzymes: ornithine decarboxylase (ODC) or agmatine deiminase (AgDI) and tyramine, histamine and cadaverine are products of decarboxylases activities, respectively, tyrosine decarboxylase (TyrDC), histidine decarboxylase (HDC) and lysine decarboxylase (LDC) (Smit, 2008, Coton *et al.*, 2010). The activity of these enzymes, with the consequent production of BAs, is a mean (1) to obtain energy (Molenaar *et al.*, 1993, Konings *et al.*, 1997, Abe *et al.*, 2002), (2) of defence against stress (Schiller *et al.*, 2000), (3) to crosstalk between bacteria (Sturgill & Rather, 2004) and (4) of raising the pH in the extracellular medium, favouring the survival of the bacteria prone to grow in less acidic environments (Lonvaud-Funel, 2001, Schelp *et al.*, 2001, van de Guchte *et al.*, 2002, Anlı & Bayram, 2008). Bacteria involved in malolactic fermentations are known to possess the above referred enzymes and can be responsible for the production of these BAs in wine. *Oenococcus oeni, Lactobacillus sp.* and *Pediococcus sp.* have been referred as the main bacteria responsible for the presence of BAs in advanced stages of wine production (Arena & Manca de Nadra, 2001, Guerrini *et al.*, 2001, Moreno-Arribas *et al.*, 2003, Landete *et al.*, 2005, Lucas *et al.*, 2005, Nannelli *et al.*, 2008). However due to the complexity of the bacterial ecosystem, studies on the production of BAs by other bacteria (non-LAB and non AAB) are still quite rare (Helinck *et al.*, 2013). The fact that the ability to decarboxylate amino acids is not widely spread among bacteria and that BAs appear in wines in an apparent random pattern are noteworthy. Furthermore, the ability for spreading this capacity has been recently revealed because horizontal gene transfer can happen, as genes responsible for the encoding of these enzymes are located in plasmids (Ladero *et al.*, 2011). Consequently, the potential for BAs production is a strain dependent and not a species dependent characteristic (Marcobal *et al.*, 2006, Inês *et al.*, 2009, Romano *et al.*, 2012). Therefore, the bacterial potential to produce BAs is extremely variable, being dependent not only on the presence of these genes in the genome but also in the surrounding microbiome. All of this makes it unpredictable to control the production of biogenic amines in wine.

Some amines are normally present in the grapes and their levels could depend on biogeography, climate and residual microbial population in the vine plant (Anlı & Bayram, 2008). Acidity of wine pH, optimal growth temperature and low concentration of SO_2 are factors that can influence positively the growth of undesirable BAs producing bacterial strains in wine. The duration of skin maceration in wine production may also influence the levels of BAs due to enrichment in amino acids and proteins, both precursors of amines. Furthermore, yeasts may also favour the production of BAs as they can also release BAs precursors to the extracellular medium, as consequence of their metabolism (Anlı & Bayram, 2008). Information regarding plasmid location of genes responsible for BAs

enzymes and of grape associated bacterial community (concerning phylogeny, biogeography and potential for BAs production) could provide answers for the arbitrary appearance of BAs in wines**.**

The production of wine without any BA is extremely difficult, or even impossible. However, by controlling the manufacturing conditions and knowing grape and vine bacteriome it is possible to reduce their levels (Anlı *et al.*, 2004).

1.2.1. Putrescine

Putrescine is a low-molecular-weight nitrogenous base, also designated 1,4-diaminobutane, and can be grouped within the aliphatic biogenic amines and the polyamines, which are molecules that contain two or more amino groups (Smith, 1981, Bardócz *et al.*, 1993). As referred above, BAs are essential for many physiological processes. Putrescine is involved in the synthesis of other polyamines, as spermidine and spermine, in cell growth and cell proliferation, in membrane fluidity, namely in erythrocytes and also, in animal cells in the stabilization of membrane skeleton. It has also been demonstrated its role in the regulation of nucleic acids structure and protein synthesis (Santos, 1996, Hou *et al.*, 2001, Wunderlichová *et al.*, 2014), as it can affect the shape of the three dimensional structure of DNA, by forming polyamine aggregates (Di Luccia *et al.*, 2009, Wunderlichová *et al.*, 2014). Humans can obtain putrescine from three different sources: (1) endogenous biosynthesis in the cells; (2) by food intake and (3) through production by the intestinal microbiota (Wunderlichová *et al.*, 2014). Normally, putrescine is used for physiologic processes and the excess is excreted by the organism; but when the intake in food is excessive it can lead to toxicity (Bardócz *et al.*, 1993, Wunderlichová *et al.*, 2014).

Toxicological effects of putrescine are not as severe as those of histamine, but putrescine can enhance the toxicological symptoms of these other biogenic amines up to 10 fold (Lehane & Olley, 2000), mainly due to the fact that putrescine inhibits the degrading enzymes of histamine (Stratton *et al.*, 1991, Hernández-Jover *et al.*, 1997, Emborg & Dalgaard, 2008). Another noxious effect of putrescine is its carcinogenic potential. Putrescine can react with nitrites, commonly present in food, (ten Brink *et al.*, 1990, Shalaby, 1996, Kalač *et al.*, 2005) and by its role on cell growth and proliferation, trigger the formation of neoplasms (Wunderlichová *et al.*, 2014).

Bacteria can produce putrescine by two metabolic pathways: (1) from ornithine by Ornithine Decarboxylase (ODC) (EC 4.1.1.17; ODC pathway) encoded by *odc* gene or (2) from agmatine by Agmatine Deaminase (AgDI) (EC 3.5.3.12; AgDI pathway) encoded by *agdi* gene (Fig. 2). Quite often, in bacteria, these two pathways can work at the same time (Tabor & Tabor, 1972, Cunin *et al.*, 1986). However, it has been described that, in wine, the majority of putrescine producing bacteria uses the enzyme ODC. (Romano *et al.*, 2012). In contrast, the enzyme AgDI is more likely to be found in bacteria from cheese and cider.

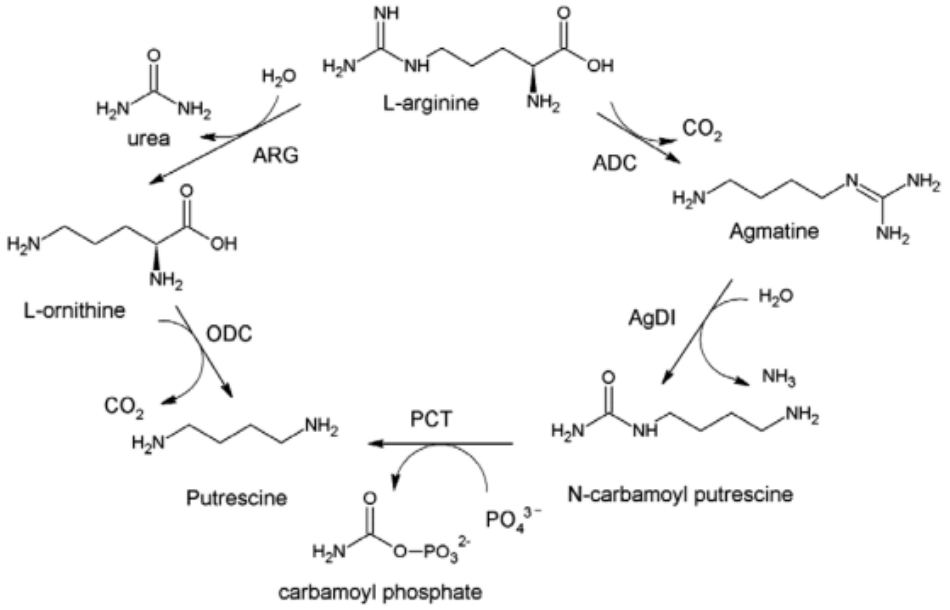


Figure 2 – Schematic approach of the different biochemical pathways that may lead to the production of Putrescine. Special focus on the Ornithine Descarboxylase (ODC) and Agmatine Deiminase (AgDI). ADC, arginine decarboxylase; ARG, arginase; PCT, putrescine carbamoyltransferase. Adapted from Wunderlichová *et al*., 2014.

The ODC enzyme decarboxylates the amino acid ornithine producing putrescine and carbon dioxide and depends on a transport protein that is responsible for the transport of amino acids into the cytoplasm, their decarboxylation and release of the putrescine; this will lead to a proton motive force and an alkalization of the cytoplasm (Romano *et al.*, 2012). On the other hand, the agmatine pathway implies two different enzymes: agmatine deiminase and putrescine carbamoyltransferase. The first transforms agmatine into N-carbamoyl putrescine and ammonia, as the second produces carbamoylphosphate and putrescine. In some strains, the AgDI pathway may occur together with the TyrD pathway in LA bacteria, as genes of both pathways are linked (Lucas *et al.*, 2007).

Even though putrescine is not considered to be one of the most toxic BAs, it is necessary to control and attenuate its levels in wine as it is linked to some health risks as well as decrease of wine quality.

1.2.2.Cadaverine

Cadaverine is the least found BA in food products, although, in low levels, can be commonly found in wine, especially in the ones produced in poor sanitary conditions (Leitão *et al.*, 2005, Del Prete *et al.*, 2009, Coton *et al.*, 2010). It is not considered to be toxic to humans (Anlı & Bayram, 2008). However, this amine, as putrescine, is also a diamine, which can interfere with the detoxification metabolism of other BAs as histamine, potentiating its toxicological effects (ten Brink *et al.*, 1990, Straub *et al.*, 1995, Landete *et al.*, 2007). Furthermore, it may also react with nitrite and form carcinogenic molecules, named nitrosamines (Santos, 1996).

Cadaverine is formed by decarboxylation of the amino acid lysine; this reaction is performed by lysine decarboxylase (LDC, EC 4.1.1.18) (Fig.3) (Landete *et al.*, 2007, Coton *et al.*, 2010), encoded by *ldc* gene.

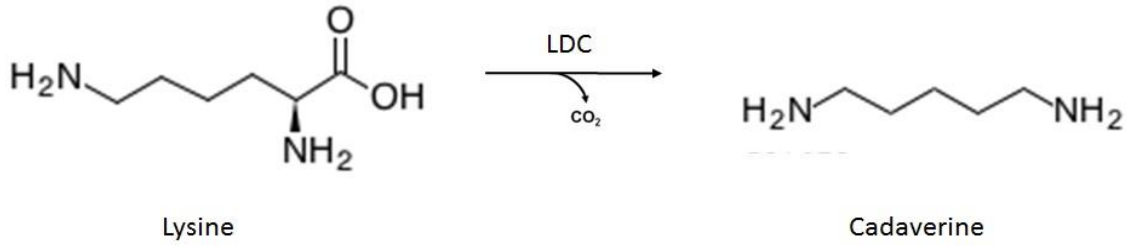


Figure 3 – Scheme of the enzymatic reaction of the Lysine Decarboxylase (LCD).

The levels of cadaverine in wine should also be controlled because it affects wine production through the modification of its organoleptic characteristics (Smit, 2008) .

1.2.3. Histamine

The monoamine histamine, however normally present in human cells, like mast cells and basophils (Karovičová, 2003), it is the most toxic biogenic amine detected in food (Santos, 1996, Fernández *et al.*, 2006, Inês *et al.*, 2009). Histamine is physiologically active by binding to specific receptors in cellular membranes. These receptors, named H1, H2 and H3, can be present in the cardiovascular, respiratory, gastrointestinal, immunological systems and skin, which implies that the negative effects of histamine can be well spread through the human body, but especially with cardiovascular repercussions (Karovičová, 2003). Dilatation of peripheral blood vessels, capillaries and arteries, and the resulting outcomes as hypotension, flushing, and headache are the main symptoms of poisoning by histamine. By binding to the H receptors, which are connected to the contraction of intestinal smooth muscle, histamine provokes abdominal cramps, diarrhea and vomiting (Taylor *et al.*, 1978, Stratton *et al.*, 1991, Santos, 1996).

The toxicological effects of the histamine depend on its concentration, presence of other BAs, amine oxidase activity and physiology of the individual. The amine oxidases,

specially monoamine oxidase (MAO) and diamine oxidase (DAO) are enzymes that are important for the detoxification of BAs by the organism. The toxicity of histamine depends on the effectiveness of these enzymes which vary between individuals, and is higher among sensitive humans or humans with deficiency in BAs detoxification metabolism (ten Brink *et al.*, 1990). Moreover, other BAs as tyramine, putrescine and cadaverine increase the absorption of histamine by the gastrointestinal tract by inhibiting MAO and DAO degrading enzymes, constraining the detoxification of the histamine and by competing for binding sites in the intestine (Kanny *et al.*, 2001, Jansen *et al.*, 2003, Karovičová, 2003, Inês *et al.*, 2008, Inês *et al.*, 2009). Likewise, wine alcohol and acetaldehyde can also increase the negative effects of this BA, which enhances the importance of reducing histamine in this drink (Landete *et al.*, 2005).

Histamine is a product of the action of the enzyme histidine decarboxylase (HDC) (EC 4.1.1.22) (Fig.4), with an optimal pH of 4.8. Depending on the strain, pyridoxal 5'-phosphate may or may not be referred as a co-factor even though it has been demonstrated to enhance amino acid decarboxylase activity as a co-factor (Lonvaud-Funel, 2001). The first HDC enzyme from a wine lactic acid bacterium was isolated from *Oenococcus oeni* and it has been studied ever since (Smit, 2008). The gene that codifies HDC, *hdc*A, is in the operon *hdc*AB that also codifies for the histidine/histamine transporter and for histidyl-tRNA sinthetase. These genes have been characterized in few bacteria belonging to the genus *Lactobacillus* (Lucas *et al.*, 2005, Martin *et al.*, 2005), as well as, the species *Oenococcus oeni* (Coton *et al.*, 1998, Coton *et al.*, 1998) and *Tetragenococcus muricaticus* (Inês *et al.,* 2009). It is accepted that the plasmid-encoding HDC system can be horizontally transferred as this gene operon appeared in other bacteria and, furthermore, lactobacilli can transfer a conjugative plasmid to bacteria of the same or different genera (Gevers *et al.*, 2003, Lucas *et al.*, 2005, Smit, 2008, Inês *et al.*, 2009).

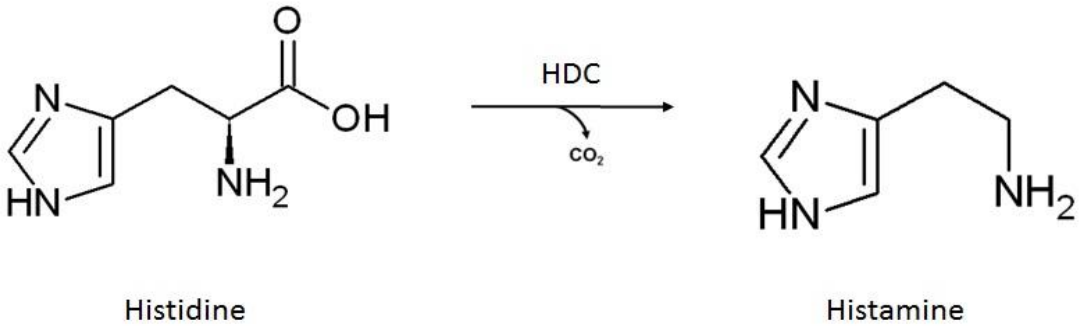


Figure 4 – Scheme of the enzymatic reaction of the Histidine Decarboxylase (HDC).

Being the most toxic BA present in food and its toxicity enhanced by other BAs as well as ethanol, the levels of histamine in wine must be controlled and its origin understood for the safety of this drink.

1.2.4. Tyramine

Tyramine is the most common biogenic amine found in cheese and can also be found in other fermented foods, like wine (Rice *et al.*, 1976, Fernández *et al.*, 2006, Fernandez *et al.*, 2006). As histamine, tyramine can be responsible for toxicological effects like increased blood pressure, urticaria, vomiting and headache (Shalaby, 1996, Fernandez *et al.*, 2007).

Tyrosine decarboxylase (TDC) (E.C. 4.1.1.25) is the enzyme which decarboxylate tyrosine into tyramine (Fig.5) (Inês *et al.*, 2009) and it is also pyridoxal 5'-phosphate dependent (Moreno-Arribas & Lonvaud-Funel, 1999, Lucas & Lonvaud-Funel, 2002). The codifying gene for TDC was sequenced in 2003 and it is constituted by four contiguous genes which encode for a tyrosyl-tRNA synthetase (*tyr*RS), the tyrosine decarboxylase (*tyr*DC), a probable tyrosine permease (*tyr*P) and a Na+/H+ antiporter (*nha*C) (Lucas & Lonvaud-Funel, 2002, Lucas *et al.*, 2003, Inês *et al.*, 2009). It has been hypothesized that the genes of tyrosine decarboxylase with the tyramine transport genes could encode for an alternative pathway, generating proton motive force; the gradient could be used by the cell for other metabolic reactions that consume energy or to generate ATP. This is a mechanism that is known to happen in other decarboxylases and supposedly it may also be present in the HDC pathway (Konings *et al.*, 1997, Christensen *et al.*, 1999, Inês *et al.*, 2009)

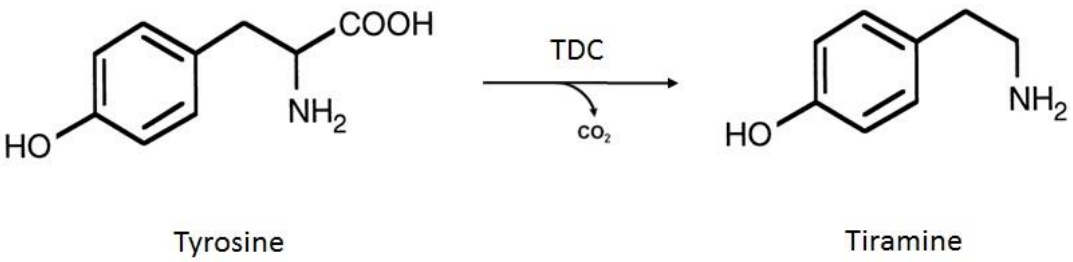


Figure 5 – Scheme of the enzymatic reaction of the Tyrosine Decarboxylase (TDC).

1.3. Methodologies used in this dissertation

1.3.1. Polymerase Chain Reaction (PCR) based techniques

In the past decades, Polymerase Chain Reaction (PCR) has become one of the most quotidian and trivial technique in laboratories around the world (Wolcott, 1992, Wagar, 1996, Elnifro *et al.*, 2000). The rapid growth and acceptance by scientific community of this methodology is due to the fact that it is a simple, sensitive, quick, highly specific technique (Landegren *et al.*, 1988, Elnifro *et al.*, 2000). In fact, it is so user friend that it is now used in many middle and high school courses (Emmanuel, 1993, Harrison, 1998, Taylor & Robinson, 1998). It allows, for instance, the analysis of the gene of the 16S rRNA which is used for taxonomic and phylogenetic identification of bacteria.

Based on this technique, the multiplex PCR is a widespread molecular biology technique for amplification of multiple target genes in a simple PCR experiment. The multiplex PCR allow us to overcome uni gene PCR setbacks as it provides the ability to amplified more than one target sequence, by using simultaneously more than one set of primers, which allows huge savings in costing and working time (Elnifro *et al.*, 2000).

The multiplex PCR has been used in a variety of areas of molecular biology as nucleic acid diagnostics (Chamberlain *et al.*, 1988, Singh *et al.*, 2006), quantitative analyses (Zimmermann *et al.*, 1996, Sherlock *et al.*, 1998), mutation and polymorphism analyses (Shuber *et al.*, 1993, Rithidech *et al.*, 1997) and RNA detection (Jin *et al.*, 1996, Zou *et al.*, 1998, Elnifro *et al.*, 2000).

In 2005, Coton and Coton (2005) has developed a multiplex PCR for the determination of potential production of BAs, more specifically histamine, tyramine and putrescine for LA bacteria, by simultaneous detection of *tyrdc*, *hdc* and *odc* genes. The multiplex PCR had proved to be fast and reliable in addition to the decrease of cost and time.

1.3.2. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a technique used for the study of microbial communities from complex samples (Muyzer *et al.*, 1995, Heuer *et al.*, 1997, Cocolin *et al.*, 2004, De Vero *et al.*, 2006, Randazzo *et al.*, 2006). Being a culture independent technique, DGGE can easily overcome the problems of culturing, like the detection of viable but nonculturable (VBNC) microorganisms. DGGE allows the analyses of PCR products with the same length but with different amino acidic conformation. The separation of these products is possible due to a polyacrylamide gel with a DNA

denaturing gradient made of urea and formamide. The primers used in PCR contain a GC-clamp in the 5' end, which stops the complete dissociation of the double-stranded DNA leading to different migrations of the DNA in the gel (Muyzer *et al.*, 1995, Muhling *et al.*, 2008, Pollet *et al.*, 2011).

After the separation of the DNA products, the profile of each sample is analysed, being assessed the position, intensity and number of bands (Zoetendal, 2001). By comparing the pattern of all samples it can easily be seen the major differences (absence/presence; intensity) of the bands and, through bioinformatic tools and statistics, it is possible to perform a more intricate analysis, like assessing phylogenetic similarity. It is also possible the excision of target bands with further amplification, sequencing and subsequent gene identification (Thakur *et al*., 2008). Furthermore, DGGE approach allows to study seasonal and spatial variations of bacterial communities (Riemann *et al.*, 1999, Riemann & Middelboe, 2002, Kan *et al.*, 2006). However, DGGE has major limitations, such as the lack of amplification of DNA from all organisms in a sample, especially those from the less represented species (König *et al.*, 2009).

DGGE studies have been used in a great variety of microbial community studies namely in grapes and wine yeasts community (Renouf *et al.*, 2005, Renouf *et al.*, 2007)

1.4. The problem in Alentejo vineyards

This dissertation appears in response to a phenomenon observed in the wine region of Alentejo: the presence of higher than expected levels of BAs in their unfermented red musts, whose origin is unknown (Leitão *et al.*, 2005). However, this problem is not present in musts produced in the Douro Superior region, despite similar environmental conditions. This situation has been identified as a potential commercial risk that requires monitoring and mitigation. A potential physiological origin due to potassium deficiency in the plant (König *et al.*, 2009) has been excluded because K levels in the vineyards are higher in Alentejo than in Douro.

It is expected that a comparative analysis between grape bacteria from the two regions will give light on the origin of this problem.

2. Objectives

The main objectives of this dissertation are:

(1) The analysis of the bacterial community present in grapes from two wine producing Portuguese regions, Douro and Alentejo. This will be achieved by (i) isolation in pure culture and the analyses of the 16S rRNA gene for isolates phylogenetic affiliation and (ii) a culture independent method, through the analysis of grape and must communities by DGGE.

(2) The analysis of the capacity of the bacterial isolates for the production of BAs. This will be achieved by the molecular analyses with single and multiplex PCR and culture experiments with BAs precursors with subsequent chemical quantification of BA production by LC-MS.

3. Material and Methods

3.1. Diagram of the methods used in this dissertation

The diagram in Figure 6 gives a comprehensive overall picture of the work performed and that is present in this dissertation.

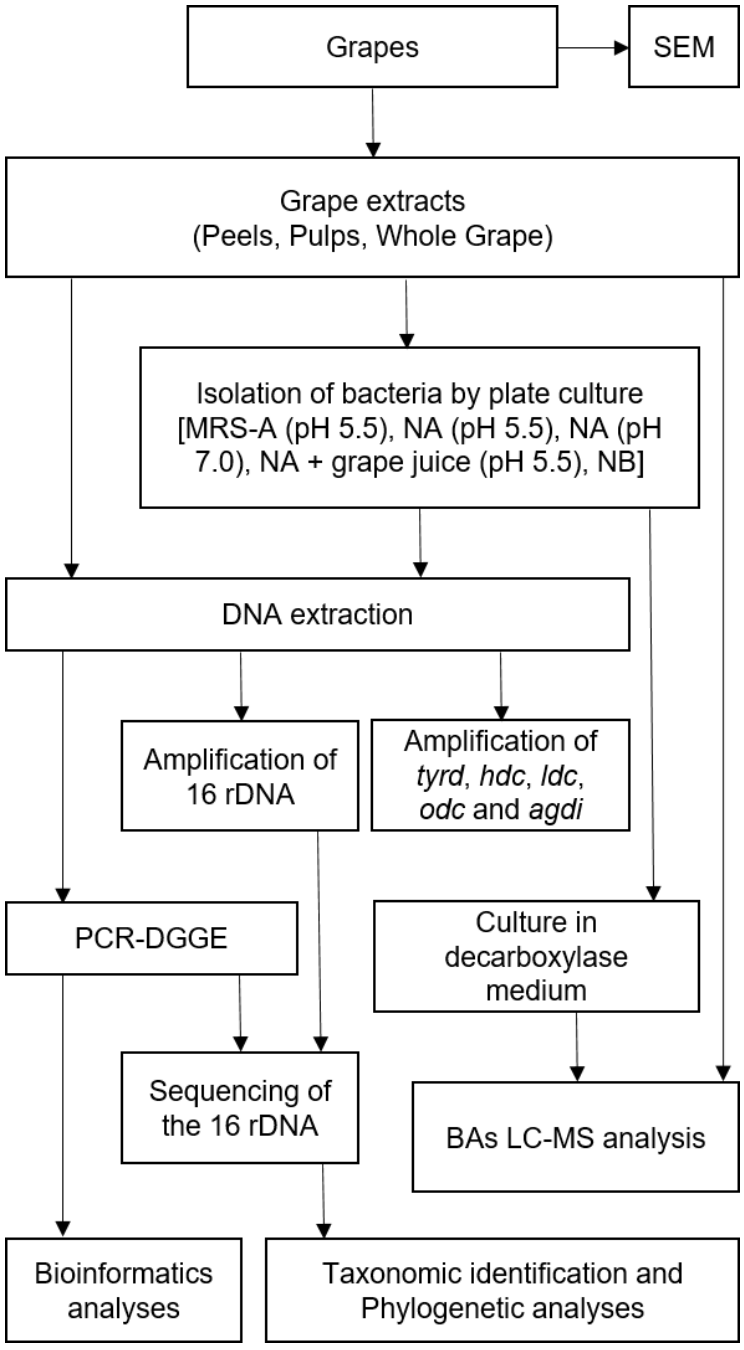


Figure 6 – Diagram of the sequenced main methodologies used in this dissertation showing the different components of the experimental design.

3.2. Sampling

Wine grapes were sampled at two different vineyards from two Portuguese wine regions, Douro Superior and Alentejo. Sampling was performed at two distinct grape maturation timings, one month before and at the harvest time. Two cultivars of *Vitis vinifera,* Alicante Bouschet (PRT53808) (Alentejo) and Touriga Franca (PRT52205) (Douro), were studied. The grapes were collected aseptically, at random spots in the vineyards, and transferred into separate sterile plastic bags. The samples were immediately transported, under cold conditions, to *Laboratório de Ecofisiologia Microbiana da Universidade do Porto* (LEMUP) and processed within 24 hours. Each sampling spot consists of three consecutive vine plants of a vine row and each sample entailed approximately 300 berries. Two and five different rows were sampled from Douro and Alentejo, respectively. Furthermore, samples from musts obtained immediately after the harvest and one week after from both sites were also collected in 2 mL tubes and frozen at -20 ºC before transported to the laboratory. Description and designation of the samples are referred in Table 2.

Table 2 – Designation of all samples used and their relation to the wine region, wine cultivar and sampling time.

Sample	Description (Region, Cultivar, Sampling time)
D(1)	Douro, Touriga Franca, Grape, During Maturation
D(2)	Douro, Touriga Franca, Grape, At the harvest
A(1)	Alentejo, Alicante Bouschet, Grape, During Maturation
A(2)	Alentejo, Alicante Bouschet, Grape, At the harvest
D(M)	Douro, Touriga Franca, Must
A(M)	Alentejo, Alicante Bouschet, Must

3.3. Isolation of bacteria

Bacterial isolation was performed from all grape samples, but not from musts. In laboratory, the grapes were manipulated under aseptical conditions, inside a Telstar Bio II A flow chamber. To enable a differential study of the bacterial community in the main grapes parts (Whole Grape (1), Skin (2) and Pulp (3)) three different treatments were implemented before inoculation – (1) 15 grapes were macerated; (2) 15 grapes were peeled off and then the skins macerated in sterile distilled water. The added volume of H_2O matched the volume obtained in extract (1); (3) 15 grapes were sterilized in 1 % NaClO (v/v) for 10 min (Hiratsuka *et al.*, 2001), followed by three washes with sterile water; the grapes were then peeled off and the pulp macerated (Fig.7). Thereafter, from each

extract of each sample, 100 µL were spread on isolation media (Table 3). The cultures were incubated in the darkness at 25 ºC and presence of growth was checked daily. Different colony morphotypes were identified (colour, size, texture and shape) under a LEICA GZ4 dissecting microscopy and transferred to the respective isolation medium. Aliquots of pure bacterial cultures were cryopreserved in Nutrient Broth medium supplemented with 20 % glycerol at -80 ºC. Six aliquots of 2 mL of each grape extract were also cryopreserved at -80 ºC, half with 20 % glycerol.

Figure 7 – Preparation of the grape extracts. A- Skins; B- Pulp; C- Macerated of the Whole Grape.

The isolation of bacteria was carried out on LAB selective MRS-A medium (pH 5.5) and non-selective Nutrient Agar medium. The MRS-A medium used was a commercial one purchased from VWR Chemicals. Three different variation of NA media were assayed as well as NB (see Table 3). The grape juice used to supplement NA medium was prepared by maceration of 1 kg of Red Globe variety supermarket grapes into juice and then sterilized through a 0.22 µm filter. The pH of the media was adjusted to 5.5 with phosphoric acid.

Table 3 – Media composition used for the bacterial isolation from grapes.

Nutrient Broth (pH 7.0)	**Nutrient Agar (pH 7.0)**	**Nutrient Agar (pH 5.5) [1]**	**Nutrient Agar + Grape Juice (pH 5.5)[1,2]**
Peptone (0.005 % (w/V))		Peptone (0.005 % (w/V))	Peptone (0.005 %)
Yeast Extract (0.003 % (w/V))		Yeast Extract (0.003 % (w/V))	Yeast Extract (0.003 %)

[1]pH was adjusted with phosphoric acid
[2] Filtrated through a 0.22µm filter

-	Agar (0.02 % (w/V))	Agar (0.02 % (w/V))	Agar (0.02 %)
Purple Bromocresol (0.005 % (V/V))		Purple Bromocresol (0.005 % (V/V))	Purple Bromocresol (0.005% (V/V))
Cicloheximide (0.0001 % (w/V))[2]		Cicloheximide (0.0001 % (w/V))[2]	Cicloheximide (0.0001 % (w/V)) [2]
-		-	Grape Juice (15 % (V/V))

In order to improve the isolation probability, different strategies for bacterial isolation were assayed in the second sampling maturation time in Alentejo samples. *In loco*, the bacterial community present in the grapes was removed with swabs which were placed in sterile bags. These swabs were swabbed on the isolation media for inoculation when in the lab. Skins of grapes were also placed directly on the media. Furthermore, entire grapes and the swabs were incubated in Nutrient Broth for 3 days and then 100 µL from each resulting culture were spread on NA medium. The isolation conditions were similar to the ones referred above.

The bacterial isolates obtained were named according to the sample and treatment, as described in Table 4.

Table 4 – Designation of the bacterial isolates. The isolates were named with codes and numbered, being the numbers represented by #.

Isolate Code	Description (Region, Timing of maturation, Treatment)
D(1)_#	Douro, During Maturation, (1) (2) and (3) extracts
D(2)_#	Douro, At the harvest, (1) (2) and (3) extracts
A(1)_#	Alentejo, During Maturation, (1) (2) and (3) extracts
A(2)_#	Alentejo, At the harvest, (1) (2) and (3) extracts
A(2)_#Z	Alentejo, At the harvest, Swab in the isolation media
A(2)_#X	Alentejo, At the harvest, Swab incubated in NB
A(2)_#U	Alentejo, At the harvest, Grapes incubated in NB
A(2)_#S	Alentejo, At the harvest, Skins placed in the media

[2] Filtrated through a 0.22µm filter

3.4. Molecular analyses

3.4.1. Grape Bacterial community DNA extraction and amplification of the 16S rRNA gene

The genomic DNA of the isolates from the grapes were extracted using the Bacterial DNA Kit (OMEGA), according to the manufacturer's instructions. The taxonomic identification of bacteria isolated was based on the analysis of the 16S rRNA gene. This gene was amplified from the extracted DNA with the universal primers 27f and 1492r (Lane *et al.*, 1991) (Table 5) in 25 µL of PCR mixture (12.5 µL of NZYTaq 2× Green Master Mix; 11 µL of nuclease-free water; 2 µM of each primer). One µL of DNA template was used for the PCR reaction. The PCR program was performed in a MyCycler™ Thermo Cycler (Bio-Rad) thermocycler and amplification conditions comprised initial denaturing step of 5 mins at 95 ºC; 30 cycles of 1 min at 94 ºC; 1 min at 52 ºC, 90 s at 72 ºC and a final extension of 5 mins at 72 ºC. PCR products were visualized in a GenoPlex (VWR), after electrophoresis in a 1.2 % agarose gel, stained with Roti Safe™ (Roth) in 1× Tris, Acetate, EDTA (TAE) buffer (OMEGA).

Table 5 – Primers used in this work in the different molecular assays.

Primer	Sequence (5' - 3')	Amplicons size (bp)	Target Gene
27f	AGAGTTTGATCMTGGCTCAG	1465	16S rRNA
1492r	ACCTTGTTACGACTT		
GC-358F	CCT ACG GGA GGC AGC AG	550	16S rRNA
907r	CCG TCA ATT CMT TTG AGT TT		
MDPQQRf	RTR GAY CCN CAG CAI CG	70	*PKS-I*
HGTGTr	VGT NCC NGT GCC RTG		
DKf	GTG CCG GTN CCR TGN GYY TC	1000	*NRPS*
MTr	GCN GG(C/T) GG(C/T) GCN TA(C/T) GTN CC		
TD2	ACA TAG TCA ACC ATR TTG AA	1133	*tyrd*
TD5	CAA ATG GAA GAA GAA GTA GG		
HDC3	GAT GGT ATT GTT TCK TAT GA	435	*hdc*
HDC4	CCA AAC ACC AGC ATC TTC		
ODC1	NCA YAA RCA ACA AGY NGG	900	*odc*
ODC2	GRT ANG GNT NNG CAC CTT C		
AgD1	CAY GTN GAY GGH SAA GG	600	*agdi*
AgD2	TGT TGN GTR ATR CAG TGA AT		
BSF8	AGA GTT TGA TCC TGG CTC AG	1537	16S rRNA
BSF1541	AAG GAG GTG ATC CAG CCG CA		

CAD1-R	TTY GAY WCN GCN TGG GTN CCN TAY AC	1098	*ldc*
CAD1-F	CCR TGD ATR TCN GTY TCR AAN CCN GG		
CAD2-R	CAY RTN CCN GGN CAY AA	1185	
CAD2-F	GGD ATN CCN GGN GGR TA		

3.4.2. Identification and phylogenetic analysis of bacteria

The PCR amplification products were purified using illustra™ GFX™ PCR DNA Kit and Gel Band Purification Kit (GE Healthcare) and Sanger sequenced by GATC Biotech. The sequences were edited and checked manually using CHROMAS 2 (Goodstadt & Ponting, 2001) correcting possible errors in chromatograms. The corrected sequences were assembled and consensus of the strains was constructed in ProSeq v2.9.2.54 and confirmed in Geneious v9.1.5. Alignment of all consensus sequences was performed using MEGA 6 (Molecular Evolutionary Genetics Analysis) software, that permits to infer overtime the molecular evolutionary between genes, genomes and species (Tamura *et al.*, 2013). The construction of the phylogenetic tree was performed the using calculation method maximum likelihood – ML in MEGA 6, applying General Time Reversible model and Gamma distributed with Invariant sites (G+I). The aligned sequences were compared in GenBank using a Basic Local Alignment Search Tool (BLAST).

3.4.3. DGGE fingerprinting of grape and must bacterial communities

The homogenized grapes from each sampling spot, in both sampling times, and the musts from both Douro and Alentejo were analysed regarding their bacterial communities through DGGE profiles. For the extraction of microbial genomic DNA from environmental samples, E.Z.N.A Soil DNA Kit (Omega) was used.

As the focus of this study was the bacterial communities, the primers GC-358F (with a GC-clamp at the 5´ end) and 907r, targeting the 16S rRNA gene, were used for the PCR reaction (Table 5). The reaction was performed in 50 µL of PCR mixture of 25 µL of NZYTaq 2× Green Master Mix; 0.5 µM of each primer and 5 µL of DNA as template. PCR amplification was performed in a MyCycler™Thermo Cycler (Bio-Rad), that consisted in an initial denaturing step of 94 ºC for 5 mins; 10 cycles of 1 min at 94 ºC; 1 min at decreasing temperature with each cycle starting at 65 ºC and ending at 55 ºC, 3 mins at 72 ºC; 20 cycles of 1 min at 94 ºC; 1 min at 55 ºC; 3 mins at 72 ºC and a final extension

of 10 mins at 72 ºC. PCR products were separated by electrophoresis on 1.2 % agarose gel in 1 × TAE buffer.

PCR products from each mixture were loaded on a DGGE gel and run at 60 °C at constant voltage of 65 volts for 16 hours in a DCode™ universal mutation detection system (Bio-Rad). The 6 % acrylamide gel with a linear gradient of denaturing conditions (100 % denaturant agent is 7 M urea and 40 % deionized formamide) ranged from 30 to 60 %, 40 to 70 % and 40 to 80 %, for gradient optimization. Gels were stained with SYBR® Gold Nucleic Acid Gel Stain during 1 hour in 1 × TAE buffer and visualized by UV light in a Gel Doc EZ System (Bio-Rad) with the Image Lab Software v4.0.1 (Bio-Rad).

After digitalization, DGGE gels were analysed with the QuantityOne software v4.6.9 (Bio-Rad). This software performs an optical intensity profile through each lane, detects the bands correlating them in the different lanes through their position in the gel. Matrix reports were statistically analysed using Primer software v7.0.11 (PRIMER-E Ltd, Ivybridge, UK) and cluster analysis, non-metric multidimensional scaling (nMDS), similarity percentage analysis (SIMPER), analysis of similarity (ANOSIM), all based in Bray-Curtis coefficient (Bray & Curtis, 1957), were performed. Furthermore, diversity index (Shannon-Weiner) (Shannon, 1948), Margalef richness index were calculated. Results were expressed as mean ± SD (standard deviation) and analysed by two-way ANOVA using IBM SPSS Stastica 24 software package (SPSS R Inc., Chicago, IL, USA) for windows, with origin and time used as fixed variables and Tuckey's multiple range tests were used for determining significant differences among means.

3.4.4. Bacterial biotechnological potential - search of polyketide synthase and nonribosomal peptide syntethase genes

The presence of genes involved in the production of secondary bioactive metabolites was screened in all bacteria isolated from the grapes. This study provides us with an idea of the antimicrobial potential of the bacterial community present in the grape phylosphere. Amplification of the extracted DNA was achieved with MDPQQRf and HGTGTr (Kim *et al.*, 2005) and DKf and MTr (Neilan *et al.*, 1999) primers, specific for PKS-I and NRPS genes, respectively (Table 5) in 25 µL of PCR mixture (12.5 µL of NZYTaq 2× Green Master Mix; 0.1 mM of each primer and 2 µL DNA template). The same PCR program was used for the amplification of the two genes in a MyCycler™Thermo Cycler (Bio-Rad). The amplification conditions consisted of an initial denaturing step of 5 min at 95 ºC; 11cycles of 1 min at 95 ºC; 30 s at 60 ºC and 1 min at 72 ºC, with the annealing temperature reduced by 2 ºC per cycle, followed by 30 cycles of 95 ºC for 1 min, 40 ºC for 30 s and 72 ºC for 1 min with a final extension of 10 min at 72 ºC. The PCR products were

visualized by electrophoresis for the presence of approximate 700 bp and 1000 bp size amplicons, for PKS-I and NRPS respectively, in a 1.2 % agarose gel in 1 × TAE buffer, in a GenoPlex (VWR).

3.4.5. Potential for the production of Biogenic Amines by grape bacteria – molecular approach

The search of genes involved in the production of BAs embraced six different genes, responsible for four different BAs – Cadaverine, Putrescine, Tyramine and Histamine. One of the analysis was based on Coton *et al.*, 2010, who developed a multiplex PCR, which targeted four different BAs genes – *agdi*, *hdc*, *odc* and *tyrd* - allowing the simultaneous detection of fragments of various genes, using a 16S rRNA coding gene as an internal control of the reaction. This method uses the primers specified in Table 5. The multiplex PCR was performed with all the bacterial isolates extracted DNA in a 50 µL reaction (25 µL of NZYTaq 2× Green Master Mix; 2 µL of DNA; 0.8 mM of ODC1/ ODC2 and AgD1/AgD2 primers; 0.2 mM of TD2/TD5 primers; 0.12 mM of HDC3/HDC4; 0.05 mM of BSF8/BSR1541 and 10 µg/mL of BSA). The PCR program consisted in an initial denaturation step of 95 ºC of 5 min; followed by 35 cycles of 95 ºC for 5 min, 52 ºC for 1 min, 72 ºC for 1 min 30 s with a final extension at 72 ºC for 5 min, performed in MyCycler™ Thermo Cycler (Bio-Rad). PCR products were visualized in a GenoPlex (VWR), after electrophoresis in a 0.8 % agarose gel, stained with Roti Safe™ (Roth) in 1× TAE buffer.

Additionally, the presence of the *ldc* gene was also assessed by PCR technique (Landete *et al.*, 2007) using primers CAD1 (for Gram-negative bacteria) and CAD2 (for Gram-positive bacteria) (Table 5). PCR was performed in 25 µL amplification reaction mixtures containing 1 µL of template DNA, 50 mM KCl, 1 µM of each primer and 12.5 µL of NZYTaq 2× Green Master Mix. The amplifications were performed in a MyCycler™ Thermo Cycler (Bio-Rad) thermocycler, using the following cycling parameters: 10 min at 95 ºC, followed by 30 cycles of 30 s at 95 ºC, 30 s at 53 ºC, and 2 min at 72 ºC, and a final step of 20 min at 72 ºC. After electrophoresis in a 1.0 % agarose gel, stained with Roti Safe™ (Roth) in 1× TAE buffer, resulting PCR products were visualized in GenoPlex (VWR).

3.5. BA production by pure cultures of bacterial isolates after growth in decarboxylase media

The strains that had demonstrated the presence of *tyrd, agdi, odc* and/ or *hdc* genes in their genomic material (section 3.4.5) were studied for their capacity to produce the

respective BA. To perform this study, the positive hits were cultivated in a specific decarboxylase medium supplemented with BAs amino acid precursors and a pH indicator (Table 6). The production of these nitrogenous compounds results in an increase of the extracellular pH leading to changes in colour, which indicates if a strain had potentially produced BAs.

The bacteria were initially pre-culture in NB, then cultivated in decarboxylase broth (Table 6) without the amino acid precursor, for 4 to 5 days (≈10^9 CFU/mL), at 30 ºC. An aliquot of the culture (0.2 mL) was inoculated into 2 mL of the same medium with and without (control) amino acid and incubated for 7 days at 30 ºC (Table 6). To test the influence of oxygen in the production of BAs by these strains, this inoculation was also performed under anaerobic conditions, by overlaying the culture with sterile paraffin. The cultures were then centrifuged to remove the cellular content at 13,300 rpm for 5 min. The supernatant was removed and maintained at -20 ºC until further analysis

Table 6 – Composition of decarboxylase media used in the chemical assays. pH was adjusted to 5.3 with phosphoric acid.

	Decarboxylase medium without amino acid (w/V)	**Decarboxylase medium for putrescine production (w/V)**	**Decarboxylase medium for tyramine production (w/V)**	**Decarboxylase medium for histamine production (w/V)**	**Decarboxylase medium for cadaverine production (w/V)**
Tryptone	0.005 %				
Beef Extract	0.008 %				
$MgSO_4$	0.0002 %				
$MnSO_4$	0.00005 %				
$FeSO_4$	0.00004 %				
$CaCO_3$	0.0001 %				
Yeast Extract	0.004 %				
Tween 80	0.0005 %				
L-histidine monohydrochloride	-	-	-	0.50 %	-
L-ornithine monohydrochloride	-	0.50 %	-	-	-
L-tyrosine	-	-	0.50 %	-	-
L-lysine dihydrochloride	-	-	-	-	0.50 %

Bromocresol purple	0.00006 %
Pyridoxal-5-phosphate	0.005 %

3.6. Chemical analysis of BAs by Liquid Chromatography – Mass Spectrometry

The following analyses were performed in Chemistry Department of the Faculty of Sciences, University of Porto.

The determination of BAs in pulps, skins and grapes samples was performed by Liquid Chromatography – Mass Spectrometry (LC-MS) technique. A hydrophilic interaction column - HILIC Silica 2.6 µm 150 × 4.6 mm, protected by a pre-column was used; the column temperature was kept constant during the whole analysis procedure, at 30 ºC. The mobile phase was constituted by 70 % acetonitrile and 30 % 33.33 mM ammonium formate buffer, with pH 3.0 (adjusted with formic acid). The race, in isocratic mode, endured for 20 min, with a 0.5 mL/min flow. The detection and analysis of mass was performed, by a Finnigan LCQ DECA XP MAX mass detector, in Simple Race Mode (SRM) and in the transition mode of each biogenic amine (138-121 for tyramine, 89-72 for putrescine, 112-95 for histamine e 103-86 for cadaverine).

Before the analysis, each sample was diluted four times with the LC-MS mobile phase.

To quantify the BA present in each sample, a calibration curve was performed, using putrescine standard (injected in duplicated), with a concentration range between 0.02 mg/L and 2.5 mg/L.

The mean values of the concentrations were analysed T-test and one-way ANOVA using Excel for determining significant differences among means.

Similar procedures were applied to analyse the levels of BAs in the culture media in the experiment to assess BAs production by the bacterial isolates.

3.7. Scanning Electron Microscopy

The skin of fresh grapes from Douro and Alentejo was directly observed on a scanning electron microscope, model Pro X, Phenom®. A cooled sample holder was used at -17 ^{0} C.

4. Results and Discussion

4.1. Levels of Biogenic Amines in Douro and Alentejo grapes and musts

This dissertation appears in response to a problem that happens in the production of wine in the Alentejo region: the presence of high levels of BAs of unknown origin in its red musts. To confirm previous information and to obtain BAs data correspondent to the samples studied in this work, in the beginning of this project the BAs present in grapes and musts from Douro and Alentejo were quantified by LC-MS technique (Table 7 and 8, Fig.8 and 9). The analyses were performed in the Chemistry Department of Faculty of Sciences, University of Porto. Putrescine was the only BA detected in grapes from both regions (Fig. 8). In the musts, tyramine was also found but in lower levels (Fig. 9). Two different temporal grape samples, one at the harvest time and another about one month before, were analysed. When comparing these two sampling times, putrescine levels in grapes increased in Alentejo but decreased in Douro. At the maturation time, a non-significant difference existed between BAs levels in Douro (0.2776 mg/L) and Alentejo (1.2444 mg/L) (Fig. 8 and 9). Furthermore, different parts of the grapes: the skin, the pulp and the whole grape, were analysed. Putrescine was majorly present on the pulp, not in the skin and the levels of the whole grape were approximately the sum of the values obtained for the skin plus the ones of the pulp (Fig. 8 and 9). Agudelo-Romero (2013) studied the levels of polyamines, including putrescine, in grapes from different Portuguese vine types, and concluded that their levels decreased during the ripening of the fruit, due to complex interactions with other growth factors, like abscisic acid (Agudelo-Romero *et al.*, 2013). The decrease of BAs levels in Douro grapes is in agreement with Agudelo-Romero (2013) results but not the increase observed in Alentejo.

Table 7 – Concentration (mg/L) of Biogenic Amines in grape and musts from Douro region. nd- not detected.

Bioamine	**Grape** (During maturation)	**Grape** (End of maturation)	**Must** (Immediately after maceration)	**Must** (One week after maceration)
Putrescine	0.590 ± 0.328	0.278 ± 0.170	2.680	3.256
Tyramine	nd	nd	nd	1.084

Table 8 – Concentration (mg/L) of Biogenic Amines in grape and musts from Alentejo region. nd- not detected.

Bioamine	**Grape** (During maturation)	**Grape** (End of maturation)	**Must** (Immediately after maceration)	**Must** (One week after maceration)
Putrescine	0.972 ± 0.391	1.244 ± 0.580	15.736	14.1291
Tyramine	nd	nd	10.740	0.779

Comparable high levels of putrescine (up to 26.54 mg /L) and of tyramine (up to 11.32 mg/L) were reported in wine (Anlı & Bayram, 2008). It is important to highlight that putrescine is not toxic for humans but only dangerous in the presence of histamine, as this potentiates its effects, (Lehane & Olley, 2000, Emborg & Dalgaard, 2008, Wunderlichová *et al.*, 2014). However, histamine was not detected in these samples. Tyramine was found in very low quantities which are harmless and, although may induce some toxicological symptoms is not considered toxic. Furthermore, no legal recommendation for the levels of

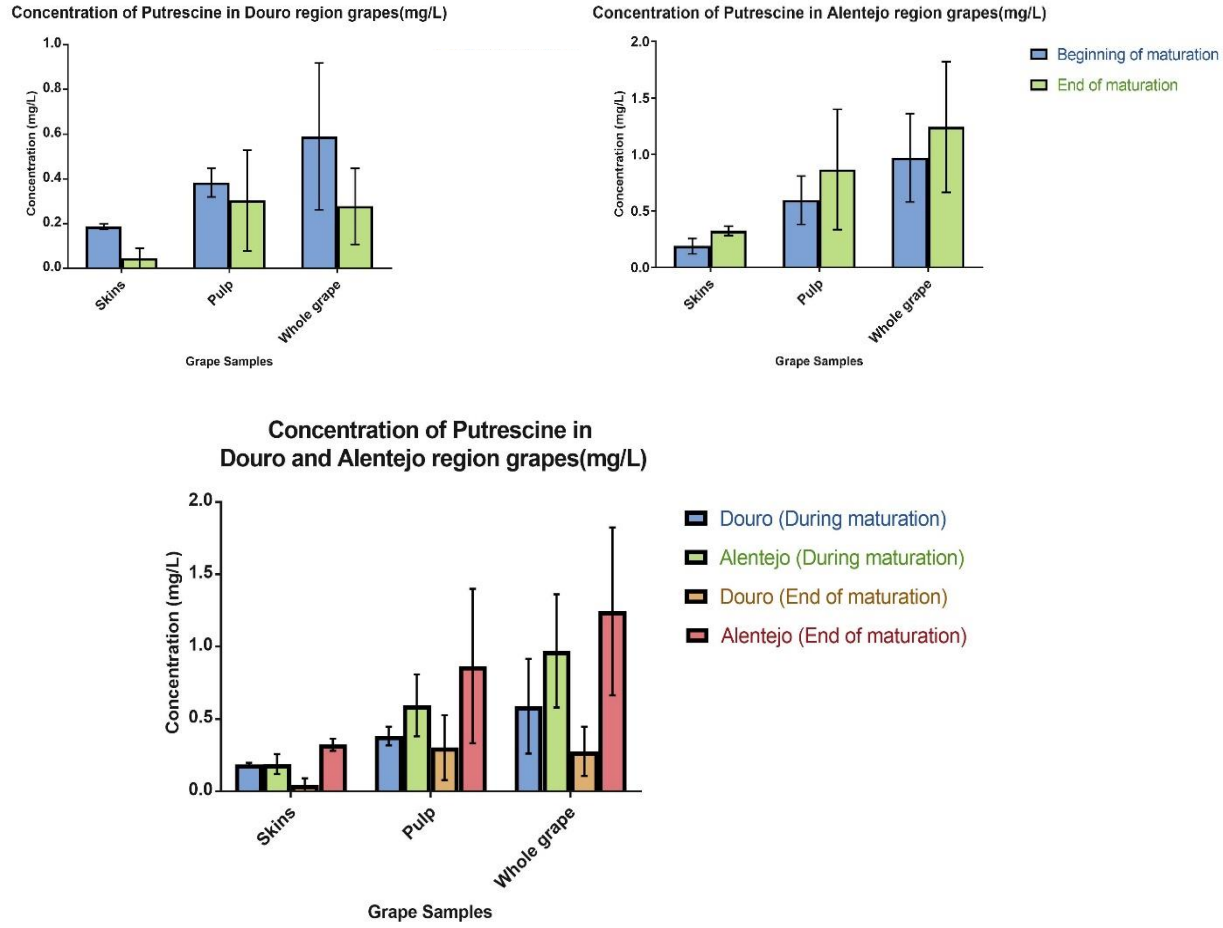


Figure 8 – Concentration (mg/L) of putrescine in different parts of Alentejo and Douro grapes, quantified through LC-MS technique. The analyses were performed in grapes sampled at the end of maturation (at the harvest) and one month before. A- Concentration of putrescine in Douro grape samples; B- Concentration of putrescine in Alentejo grape samples; C- Concentration of putrescine in Douro and Alentejo grape samples.

these two amines in food exists, nor have been proved to be dangerous (Leitão *et al.*, 2000, Leitão *et al.*, 2005, Soufleros, 2007).

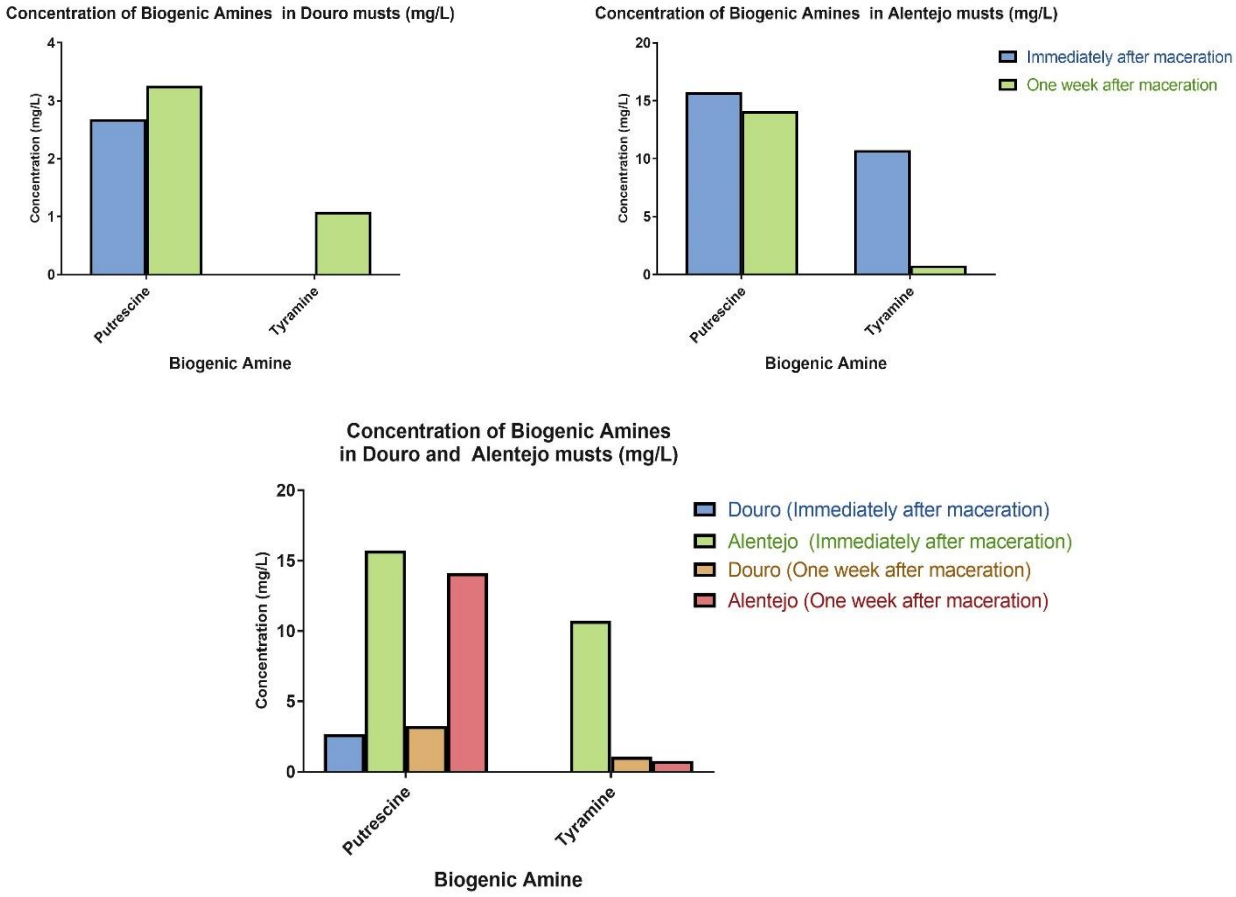


Figure 9 – Concentration (mg/L) of putrescine and tyramine in musts of Alentejo and Douro grapes, quantified through LC-MS technique. The analyses were performed in musts samples immediately after maceration and one week later. A- Concentration of putrescine and tyramine in Douro must samples; B- Concentration of putrescine and tyramine in Alentejo must samples; C- Concentration of putrescine and tyramine in Douro and Alentejo must samples.

4.2. Grape associated bacterial biodiversity

4.2.1. Bacterial isolation studies

Isolation of microorganisms from grape epiphytic bacterial community was performed in Douro and Alentejo samples. In order to optimize isolation, different strategies for bacterial isolation were assayed in the second time of sampling, at grape maturation, in Alentejo samples, like the use of swabs to collect bacteria and their incubation in NB medium.

A total of 168 isolates were obtained, 14 (8 %) from Douro and 154 (92 %) from Alentejo. However, when contemplating the isolates that were retrieved with equivalent isolation methodologies from the two regions, only 62 isolates were obtained with a

representation of 23% (n=14) and 77% (n=48) from Douro and Alentejo, respectively. These results may indicate a great influence of biogeography on grape bacteriome as was observed by Bokulich *et al.* (2014) in musts (freshly grape juice containing skin and seeds).

It is noticeable that the implementation of different methodologies of isolation on A(2) samples lead to an increase in the number of isolates (Fig. 10), and that the swab of the grapes (A(2)_#Z) was the technique which allowed to retrieved more bacteria (31% of all isolates). The greater number of isolates from Alentejo *versus* Douro is noteworthy, with differences of 11-fold or 3-fold depending if all the isolates are considered or just the ones from the same isolation techniques. An increase in the number of the bacterial grape community with the maturation of the grape was evident as, both in grapes from Douro and Alentejo, the number of isolates had an increase of about 6 %.

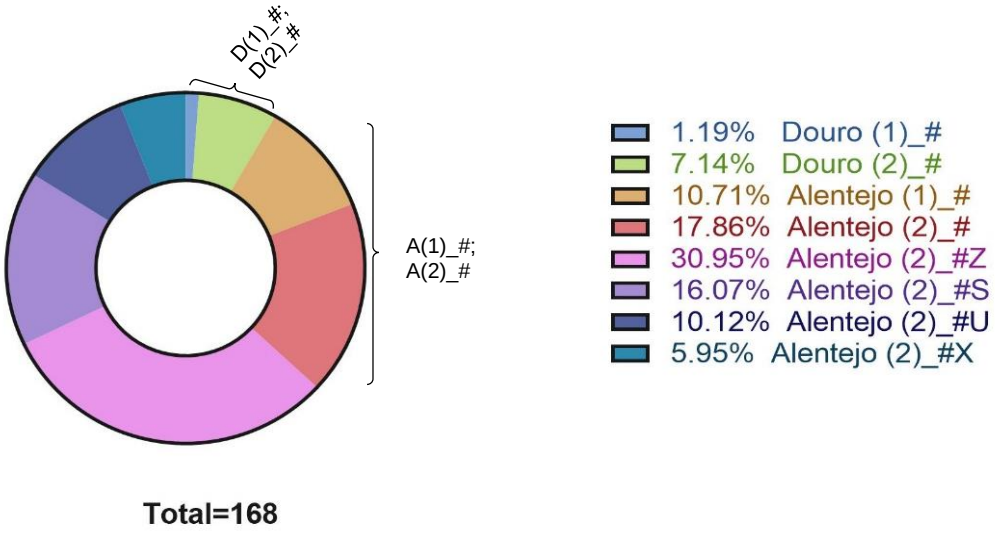
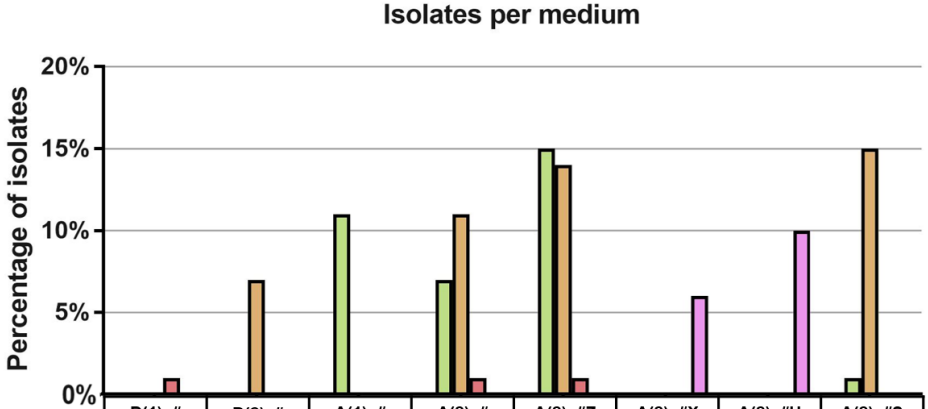


Figure 10 – Distribution of percentage of isolates obtained from each different isolation technique. (n_{TOTAL}=168; $n_{Douro(1)\_\#}$=2; $n_{Douro(2)\_\#}$=12; $n_{Alentejo(1)\_\#}$=18;$n_{Alentejo(2)\_\#}$=30; $n_{Alentejo(2)\_\#Z}$=52; $n_{Alentejo(2)\_\#S}$=27; $n_{Alentejo(2)\_\#U}$=17; $n_{Alentejo(2)\_\#X}$=10).

In this study, two different isolation media were used, in a total of five different variations. MRS-A was used because it is a selective medium for LAB, which are bacteria known to produce BAs as a result of their metabolism. This medium is commonly used for the growth of Lactic Acid wine Bacteria (De Man *et al.*, 1960, Coton *et al.*, 2010, Ladero *et al.*, 2011). The other medium, Nutrient Broth/Agar, is a widely used general purpose medium that supports the growth of many non-fastidious bacteria. Contrary to what was expected, no isolate was retrieved in the selective medium MRS-A (Fig. 11). This most probably was due to a problem of the medium formulation, as also no growth was obtained from yogurt, that is known to possess LABs. Yogurt was, thus, used as a control. However, it is known that LABs are present in grapes although in very reduced concentration and, consequently, only possible to detected many times by specific isolation techniques (Renouf *et al.*, 2005, Renouf *et al.*, 2007, Barata *et al.*, 2012). The medium with a higher

number of isolates was NA (pH 7.0) (47 %) followed by NA (pH 5.5) (34 %). Even though the neutral pH may create a more suitable environment for a higher number of bacteria, the lower pH of the medium simulates better the acidic conditions found in wine. The lower pH, is, thus, more appropriate for the isolation of wine bacteria and, consequently, for the isolation of BAs producing bacteria (Lonvaud-Funel, 2001, Schelp *et al.*, 2001, van de Guchte *et al.*, 2002, Anlı & Bayram, 2008). Supplementing NA medium with grape juice in order to simulate closer conditions to the ones present in musts, only allowed the obtainment of 3 % of the total isolates. This medium, curiously, was not as effective as the NA (pH 5.5) without supplementation (Fig. 11). Isolation in all NA media tested was only obtained for samples A(2)_# and A(2)_#Z (Fig. 11). These were also the samples where higher number of isolates were retrieved, which probably indicates the presence of a more diverse and numerous community in grapes from samples A(2) comparatively to the ones of D(2) samples.



	D(1)_#	D(2)_#	A(1)_#	A(2)_#	A(2)_#Z	A(2)_#X	A(2)_#U	A(2)_#S
MRS-A	0 %	0 %	0 %	0 %	0 %	na	na	0 %
NA (pH 5.5)	0 %	0 %	11 %	7 %	15 %	na	na	1 %
NA (pH 7.0)	0 %	7 %	0 %	11 %	14 %	na	na	15 %
NA + Grape juice (pH 5.5)	1 %	0 %	0 %	1 %	1 %	na	na	0 %
NB	na	na	na	na	na	6 %	10 %	na

Figure 11 – Percentage of isolates (n_{TOTAL}=168) distributed by sample and medium of isolation. No isolates were retrieved from MRS-A; 34% of all isolated from NA (pH 5.5); 47% from NA (pH 7.0); 3% from NA supplemented with 15 % grape juice and 16% from NB. D(1)_# - Douro, first sampling, Whole, Skins and Pulp extracts; D(2)_# - Douro, second sampling, whole grape, skins and pulp extracts; A(1)_# - Alentejo, first sampling, whole grape, skins and pulp extracts; A(2)_# - Alentejo, second sampling, whole grape, skins and pulp extracts; A(2)_#Z - Alentejo, second sampling, swab in the isolation media; A(2)_#X - Alentejo, second sampling, swab incubated in NB; A(2)_#U - Alentejo, second sampling, grapes incubated in NB; A(2)_#S - Alentejo, second sampling, Skins placed in the media. na – not assayed.

To do a comparable study between Douro and Alentejo, only samples from D(1)_#, D(2)_#, A(1)_# and A(2)_# can be analysed. Although colonization of grape vine internal tissues by endophytes is well establish (Compant *et al.*, 2011), no isolation was obtained from pulp extracts (Fig. 12), which is indicative of an absence of bacterial community inside the grapes. From both Whole Grape and Skin extracts, 62 bacteria were isolated (see Fig. 12). The number of isolates obtained from the Whole Grape extract was slightly higher

than the ones from the Skin extract. The different number obtained between these two extracts may be related to the sugars or other nutritional factors presents in the pulp, that could be used as an additional source of nutrients, favouring the growth of bacteria in the medium after inoculation of the Whole Grape extracts. None of the isolation media seems

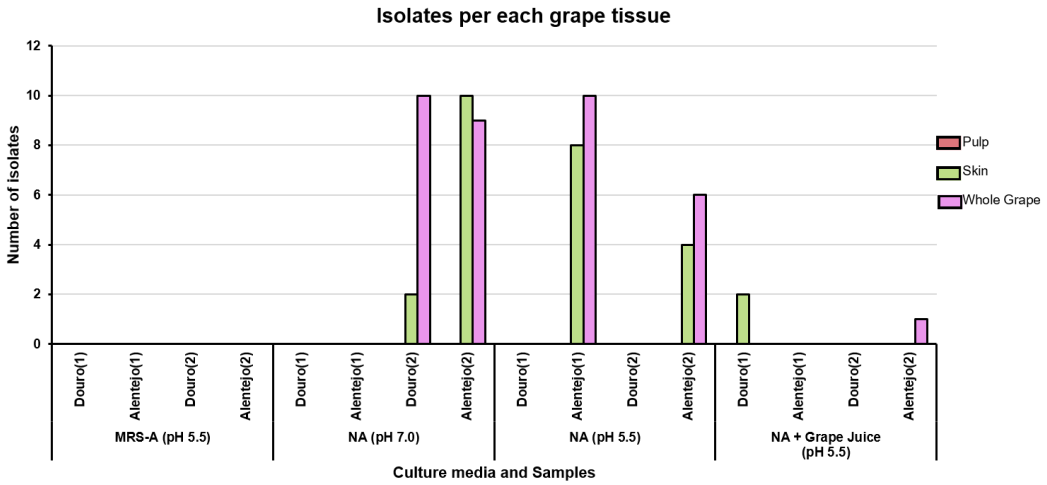


Figure 12 – Number of isolates retrieved from Alentejo and Douro grapevines distributed per sampling, culture media and location within the grape ($n_{Total\ of\ isolates}$ = 62). Bacteria were not able to grow in MRS – A medium. NA (pH=7.0) was the media that had more number of isolates (n=31; 50%). No isolates were obtained from pulp. Whole Grape was the extract with more isolates (n= 36; 58%). A higher number of bacteria was retrieved from Alentejo extracts (n = 48; 77 %), especially from the second sampling (n= 29; 47%), comparatively to the Douro ones.

to have favoured isolation in any of the different grape parts tested. Moreover, in NA (pH 5.5) only bacteria from Alentejo grew and in NA (pH 7.0) only were obtained isolates from the second sampling. The reason behind the absence of bacterial isolation in Douro in NA (pH 5.5) and in Douro (1) and Alentejo (1) in NA (pH 7.0) is not understandable.

4.2.2.Phylogenetic analysis of grapes bacterial isolates

The phylogenetic analysis after the 16S rRNA being Sanger Sequencing and 80 bacteria were identified of the 168 isolates (Fig. 14, 15 and 16; Annex 1). Figure 13

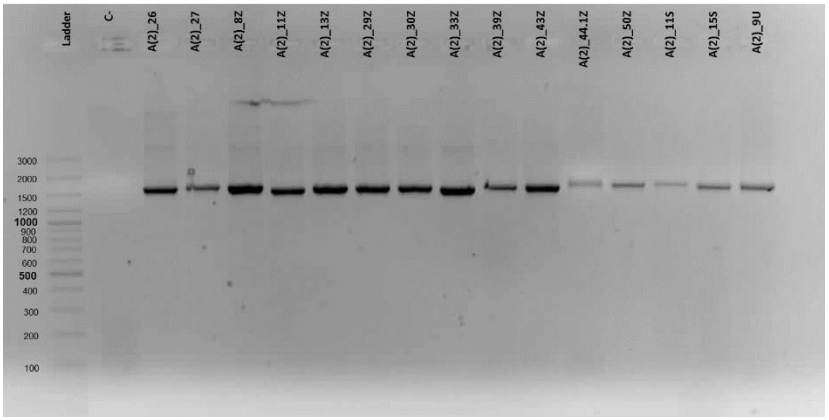


Figure 13 – Electrophoretic agarose gel evidencing the PCR products of 16S rRNA gene amplification from Alentejo isolates. The used Ladder was MR17 DNA Ladder (DNAgdansk); C- stands for Negative Control of the PCR reaction. For isolates designation see Table 4.

demonstrates the amplification of the 16S rRNA of various isolates. Some of these isolates lost the capacity to grow after sub culturing and, from some, no DNA could be obtained. This justifies the lower bacterial numbers used in various subsequent studies.

In this study, the phylum with a higher number of isolates was *Firmicutes* (56 %) followed by *Actinobacteria* (27%), *Alphaproteobacteria* (14%) and *Gammaproteobacteria* (3%) (Fig. 14). Overall, of 80 sequenced isolates we recovered 67 different strains (Annex 1). Few isolates were duplicates of the same organism and, thus, we obtained 17 (out of 21) different *Actinobacteria*, 8 (out of 11) different *Alphaproteobacteria*, 39 (out of 45)

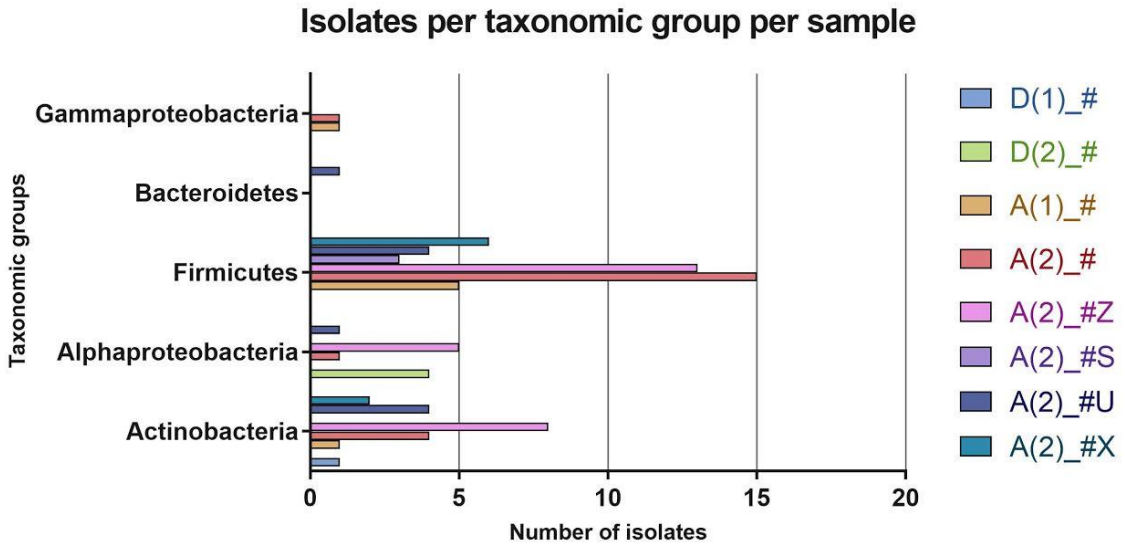


Figure 14 – Number of obtained isolates from each different phyla and their sample origin. 56 % of total of isolates are *Firmicutes* (n= 46) [of those being 5 from A(1)_#, 15 from A(2)_#, 13 from A(2)_#Z , 3 from A(2)_#S, 4 from A(2)_#U and 6 from A(2)_#X]; 23 % of isolates are *Actinobacteria* (n=20) [being 1 from D(1)_#, 1 from A(1)_#, 4 from A(2)_#, 8 from A(2)_#Z, 4 from A(2)_#U and 2 from A(2)_#X], 13 % of total of isolates are *Alphaproteobacteria* (n=11) [belonging 14 to D(2)_#, 1 from A(2)_#, 5 from A(2)_Z and 1 to A(2)_#U]; 2 % of total of isolates are *Gammaproteobateria* (n =2) [1 from A(1)_# and 1 from A(2)_#] and 1% of total of isolates are *Bacteroidetes* (n=1) [from A(2)_#U]. For isolates designation see Table 4.

different *Firmicutes*, 2 (out of 2) different *Gammaproteobacteria* and 1 *Bacteroidete* (Annex 1). We obtained 21 different genera from the epiphytic community of grapes. Our numbers are considerably higher than the ones obtained by Martins *et al.* (2013) that only obtained 9/10 genera. Differently from our results, other authors described that *Proteobacteria* was the group with a higher number of isolates (Barata *et al.*, 2012, Gilbert *et al.*, 2014, Pinto *et al.*, 2014, Zarraonaindia *et al.*, 2015, Pinto & Gomes, 2016). However, some of these studies were culture-independent, which gives a more complete picture of the bacterial diversity of the grape without the limitations of the culture-dependent method.

In our study, NA media seemed to favour the growth of many *Firmicutes* especially *Bacilli.* Members of this class are one of the most commonly found on grapes (Barata *et al.*, 2012, Gilbert *et al.*, 2014, Pinto *et al.*, 2014, Zarraonaindia *et al.*, 2015). Several were

the *Bacillus* species obtained in our study that are related to grape vine, other plants or soil environment (Martins *et al.*, 2013). *Bacillus megaterium* is used in agriculture and horticulture to solubilized natural phosphates present in soil in order to make it available for the plant. This bacterium is known to be a vine endophyte species as well as a soil bacterium (Barata *et al.*, 2012, Martins *et al.*, 2013, Baldan *et al.*, 2014, Gilbert *et al.*, 2014, Pinto *et al.*, 2014, Zarraonaindia *et al.*, 2015). *Bacillus vallismortis* was originally isolated from Death Valley soil (Roberts *et al.*, 1996). *Bacillus methylotrophicus* is a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil (Madhaiyan *et al.*, 2010). Other *Bacilli* retrieved were *Lysinibacillus fusiformis* originally isolated from the surface of the plant *Beta vulgaris* and known from various environments which include farming soil (He *et al.*, 2011). *Paenibacillus polymyxa* was isolated from roots nodules of bluepea and can be found in a variety of environments such as soils and the rhizosphere of plants where it has effects against deleterious microorganisms (mainly fungi). Furthermore, it can invade plant roots and form biofilms in a symbiotic relationship (Timmusk *et al.*, 2005). Members of *Paenibacillus* were already identified in *Vitis vinifera* (Baldan *et al.*, 2014). Although normally present in wine, only one LAB bacterium, *Pediococcus pentosaceus,* was isolated in our study. This result may be due to the absence of isolation in the selective MRS-A medium that was the medium dedicated for the isolation of this bacteria. *Staphylococcus* species were already described associated to the bacterial grape community (Barata *et al.*, 2012, Baldan *et al.*, 2014).

Regarding *Actinobacteria*, three isolates were identified as two different *Arthrobacter sp.*. This genus is commonly found in soil samples and was described in foliage of apple orchard (Scheublin & Leveau, 2013). *Curtobacterium*, which has already been described to be present in *Vitis vinifera* (Baldan *et al.*, 2014), was the genus with two close isolates, one from Alentejo and one from Douro. Different *Dermacoccus* species have been related to soil and plant environments (Pathom-Aree *et al.*, 2006). Members of *Agrococcus*, *Microbacterium* and *Kocuria kristinae* were described in grape plant environment (Barata *et al.*, 2012, Baldan *et al.*, 2014).

The *Alphaproteobacteria Agrobacterium tumefaciens* is a soil bacteria known to induce crown gall disease in dicotyledonous plants and it is a serious pathogen of grape vines (Ma *et al.*, 1987, Szegedi, 2005, Faist *et al.*, 2016). *Methylobacterium adhaesivum* is a bacterium that can be found typically in soils, foliage, and in other parts of plants (Lidstrom & Chistoserdova, 2002). The methanol emitted by the stomata of plants is used by this bacterium and it has also been demonstrated that this microorganism can stimulate seed germination and plant development (Dourado *et al.*, 2013).

The Gammaproteobacteria Enterobacter and Acinetobacter have both been detected in grape berries (Barata *et al.*, 2012).

The only *Bacteroidetes* isolated, affiliated to *Wautersiella falsenii*, should be considered as a contaminant as it is a clinical isolate (Kampfer *et al.*, 2006, Giordano *et al.*, 2016).

Bacterial that typically are present in vineplant environment namely grapes like *Massilia, Pseudomonas* and *Sphingomonas* (Charters, 2006, Martins *et al.*, 2013, Bokulich *et al*., 2014, Zarraonaindia *et al.,* 2015) were not found which could be due to non-favourable culture conditions or to biogeography (Bokulich *et al.*, 2014). On the other hand, isolates related to genera/species not found in environments related to soils or plants like *Dietzia maris*, *Leucobacter*, *Rhodococcus*, *Brevundimonas* and *Roseomonas* were retrieved in our study.

The majority of studies on wine microbiome described the presence of specific groups of bacteria, as Acetic Acid Bacteria and Lactic Acid Bacteria, and are focused on them. One of the groups of Proteobacteria more described in wine is Acetic acid bacteria (AAB), which presence is normally related to spoiled grapes (Barbe *et al.*, 2001, Barata *et al.*, 2012). In our study no AAB isolate was recovered. This may be explained as sampling was only performed on grapes in perfect conditions.

As described above, several of bacteria genera species retrieved in this work live in soil environment which suggests, as already described (Martins *et al.*, 2013), that soil must be a source of the bacteria present in grapes. Martins *et al.* (2013) in their study of bacteriome present in soil, bark, leaves and grapes of vine plants found members of the genera *Acinetobacter* and *Paenibacillus* as being soil specific and *Staphylococcus* and *Agrobacterium* only found in soil and bark. However, isolates belonging to these genera were retrieved from grapes in our study. The proximity between grapes and the soil facilitates the migration of bacteria from the soil to the grape through rain splash, wind, insects and farming management practices (Martins *et al.*, 2013).

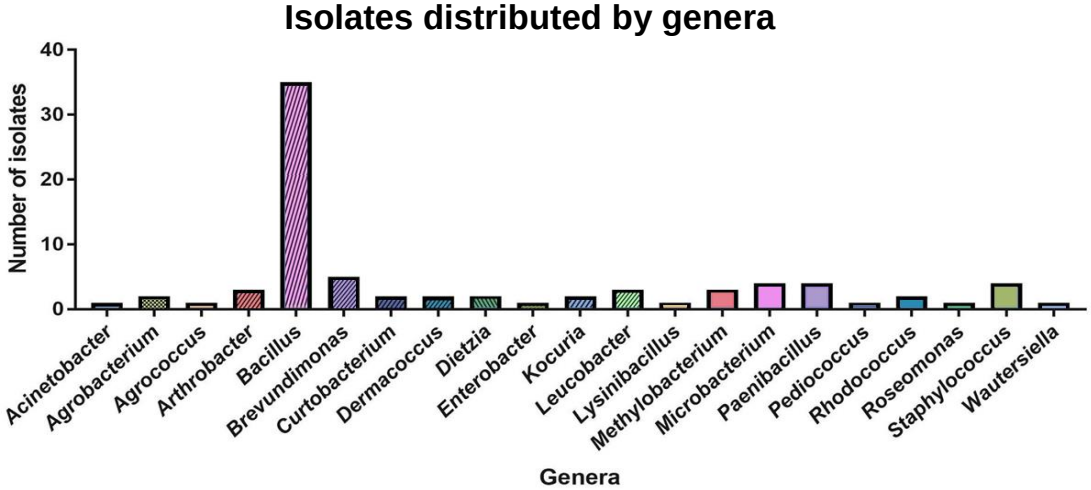


Figure 15 – Number of isolates of each genus. *Acinetobacter* – 1 isolate; *Agrobacterium* – 2 isolates; *Agrococcus* – 1 isolate; *Arthrobacter* – 3 isolates; *Bacillus* – 35 isolates; *Brevundimonas* – 5 isolates; *Curtobacterium* – 2 isolates; *Dermacoccus* – 2 isolates; *Dietzia* - 2 isolates; *Enterobacter* – 1 isolate; *Kocuria* – 2 isolates; *Leucobacter* – 3 isolates; *Lysinbacillus* – 1 isolate; *Methylobacterium* – 3 isolates; *Microbacterium* - 4 isolates; Paenibacillus - 4 isolates; *Pediococcus* -1 isolate; *Rhodococcus* – 2 isolates; *Roseomonas* – 1 isolate; *Staphylococcus* – 4 isolates; *Wautersiella* - 1 isolate.

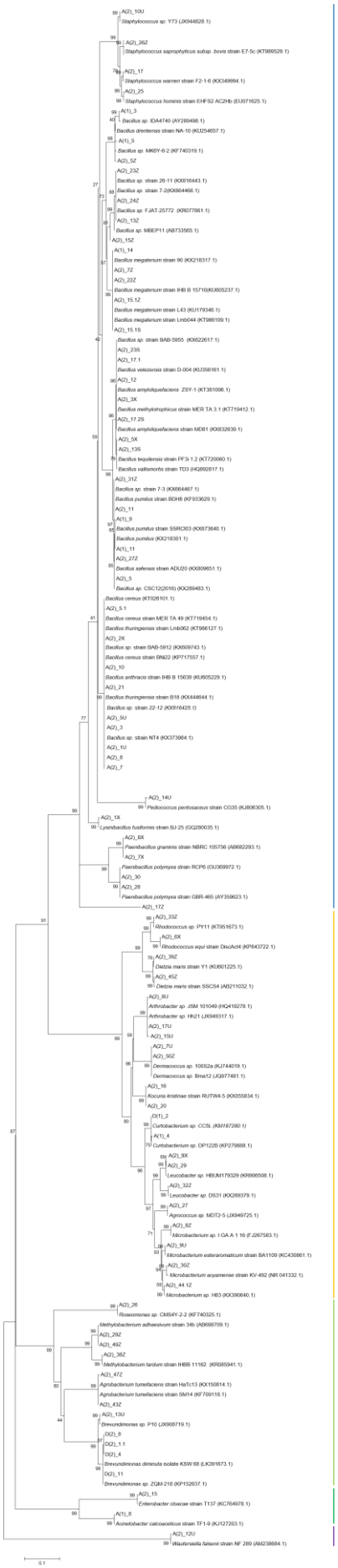


Figure 16 – Phylogenetic 16S rRNA gene tree generated by maximum-likelihood analysis based in General Time Reversible model and Gamma distributed with Invariant sites (G+I) indicating the relationship of bacterial isolates from grapes. (n $_{TOTAL}$= 68) Bar – 0.05 substitutions per 100 nucleotides. The phylogenetic analysis revealed that these isolates belong to five major groups: *Firmicutes* (56 %; n=45) *Actinobacteria* (27%; n=21), *Alphaproteobacteria* (14%; n=11), *Gammaproteobacteria* (3%; n=2)) and Bacteroidetes (1%; n=1).

4.2.3.DGGE analysis

The variation in the bacterial communities was studied by DGGE fingerprinting of grapes in different maturations stages and their initial transformation into wine. Grapes from two different wine regions, Douro and Alentejo, were compared through 3 different sampling times: at the harvest (2), one month before (1) and one week after maceration (3) of the grapes. In this analysis, musts were considered as a third timing of sampling.

For the 16S rRNA gene DGGE profiles, three different gradient concentrations were tested and the gradient 30 to 60% was the one where the band profiles were clearer and better band separation achieved (Fig. 17).

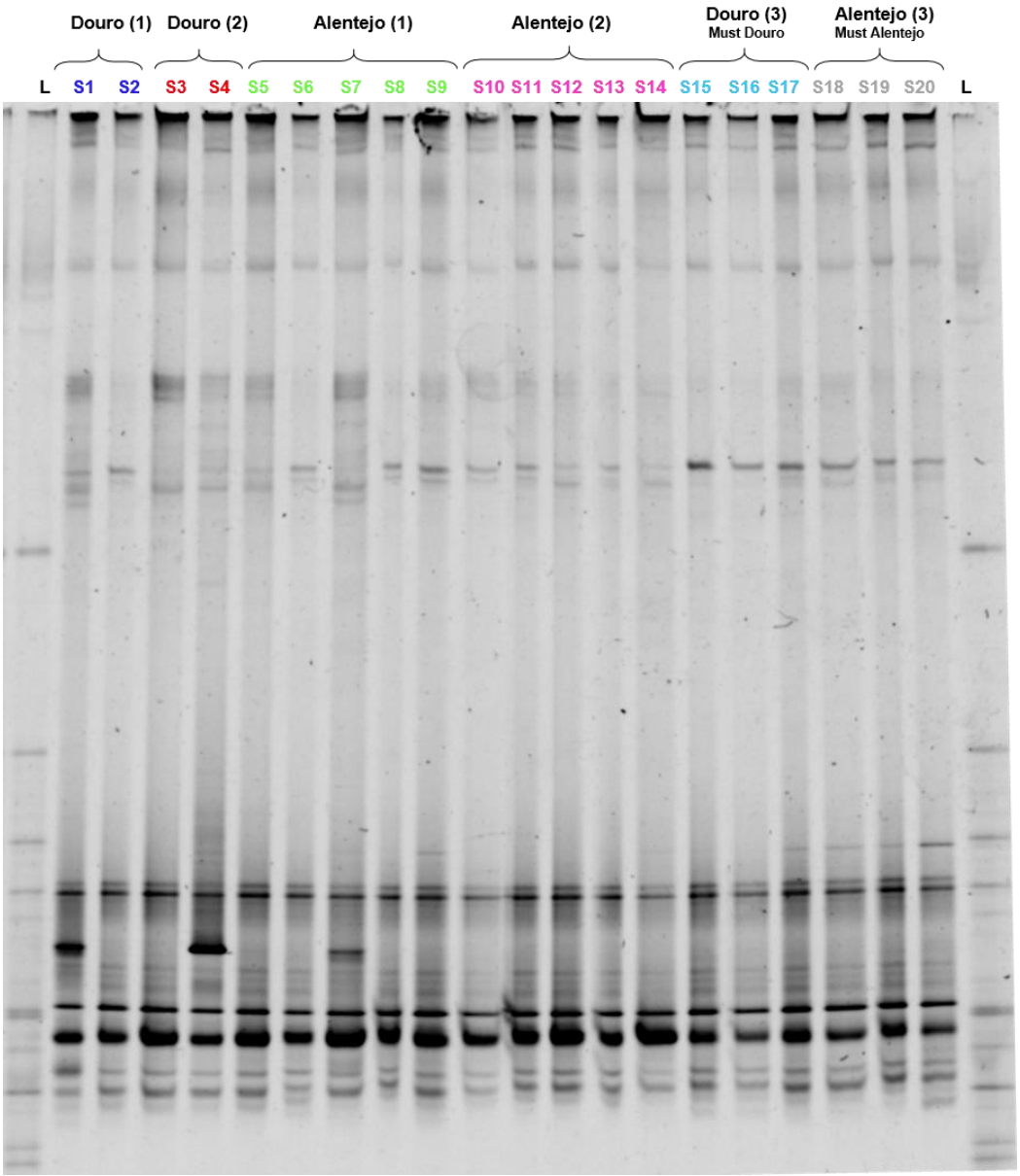


Figure 17 – Comparison of bacterial communities' fingerprinting of Grapes from Douro (1) (lane 1 and 2), Douro (2) (lane 3 and 4), Alentejo (1) (lane 5,6,7,8 and 9), Alentejo (2) (lane 10,11,12,13 and 14), Douro (3) [Must Douro] (lane 15, 16,and 17) and Alentejo (3) [Must Alentejo] (lane 18, 19 and 20) through the analysis of 16S rRNA gene DGGE gel profiles. L – Ladder, uncharacterized DNA samples. The denaturing gradient used was 30-60%.

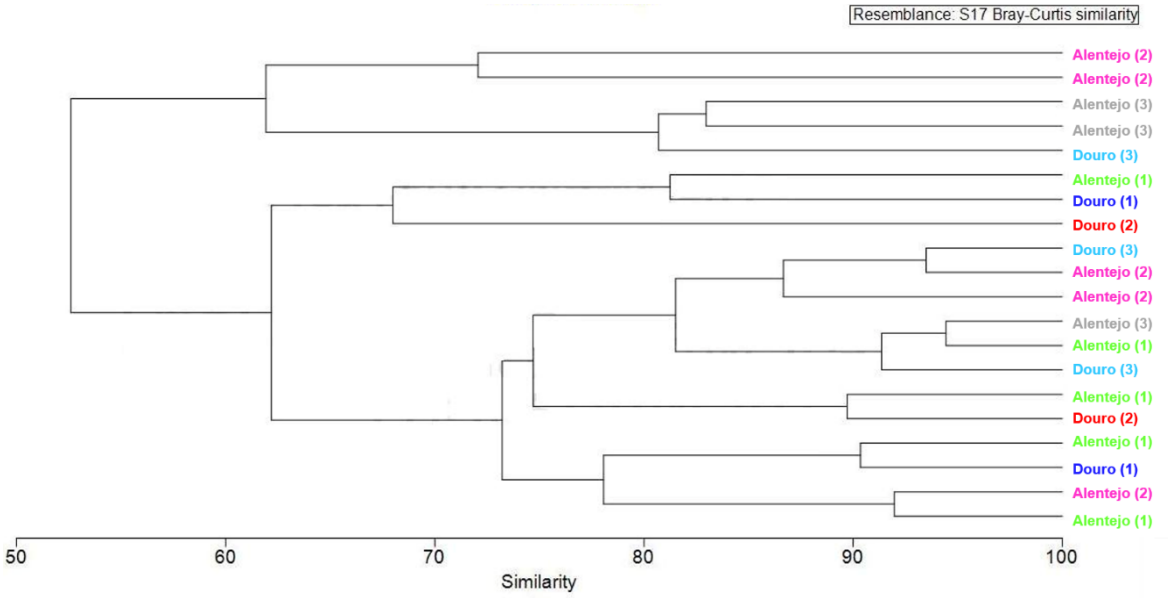


Figure 18 - Dendrogram of DGGE profiles of grapes and musts samples, based on Bray-Curtis similarity.

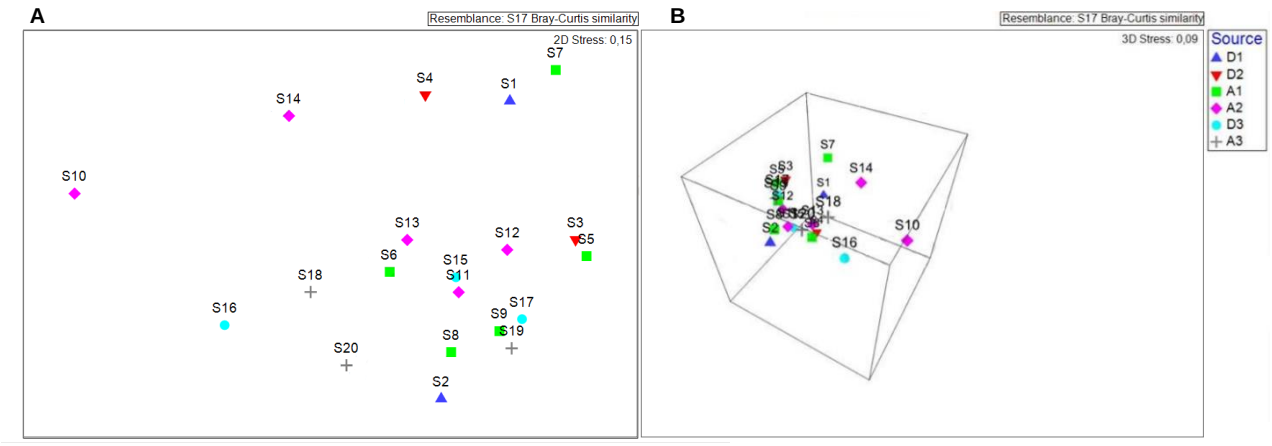


Figure 19 - Non-metric multidimensional analysis scaling (nMDS) plot based on Bray-Curtis similarity. A - nMDS using 2D (stress plot: 0.15); B - nMDS using 3D (stress plot: 0.09).

For the DGGE gel analyses, relative quantity matrices were created and used to produce a dendrogram (Fig. 18) and nMDS plots (Fig. 19), using Bray-Curtis similarity. It is clear, in the dendrogram and in the nMDS, that no differences between the bacterial communities exists as no cluster of the samples occurs. The nMDS stress value indicates how well the ordination represents the real differences of the samples. Stress values of <0.05 reflect an excellent representation with no outlook of misinterpretation; a stress value <0.1 matches to a good ordination with no real risk of taking false extrapolations; stress <0.2 can still lead to a usable picture, although there is potential of misleading, thus, too much confidence on the details of the plot should not be taken. However, an ordination with more dimensions could lead to a smaller stress value, although it could hamper the interpretation (Clarke, 1993). For this reason, after the ordination in 2-D (Fig.19A), and since the stress value was near 0.2, we performed an ordination in 3-D (Fig. 19B), which lead to a decrease in stress value to 0.09, that we accept as a good representation of the

real differences. It is noticeable that all the samples clustered together with no differences between origin or time of the samples indicating that there is no difference between these bacterial communities.

Furthermore, indexes regarding the average number of operational taxonomic units (OTUs), species richness (Margalef species richness index) and diversity (Shannons diversity index) as well as the similarity between group replicates were calculated (Table 9). Results were expressed as mean ± SD (standard deviation) and analysed by two-way ANOVA and Tukey's multiple range tests (Table 9 and table 10). There were no significant statistical differences between Douro and Alentejo bacterial grape communities (Table 10) nor among the different temporal samples. The statistical data is consisting with the results of the dendrogram and nMDS, indicating no difference between the communities.

Table 9 – Mean values regarding average number of operational taxonomic units, diversity index, species richness and similarity of the samples, calculated through Primer software.

Sampling	Douro			Alentejo		
Time	**1**	**2**	**3**	**1**	**2**	**3**
OTUs[a]	16 ± 0.0	14 ± 1.4	13.7 ± 2	14.4 ± 0.89	13.2 ±2.3	15±1
Richness[b]	0.9± 0.0	0.8 ± 0.1	0.8±0.1	0.8± 0.1	0.7 ± 0.1	0.9±0.1
Diversity[c]	2.7 ± 0.0	2.6 ± 0.1	2.5±0.1	2.6± 0.0	2.5 ± 0.1	2.6±0.1
SIMPER Similarity (%)[d]	67.8±6.7	68.8 ± 0.0	72.8±8.5	63.5 ± 2.8	73.6 ± 9.3	78.9±4.2

Values presented as means ± standard deviation (±SD); [a]OTUs: Average number of operational taxonomic units; [b]Margalef species richness: d=(S-1)/log(N);[c]Shannons diversity index: H'=-∑(pi(lnpi)); [d]SIMPER, similarity percentage within group replicates.

Table 10 – Results of the two-way ANOVA and Tukey's test regarding all group samples and variables.

Two-way ANOVA Variation source	Douro	Alentejo	Interaction
OTUs[a]	ns	ns	ns
Richness[b]	ns	ns	ns
Diversity[c]	ns	ns	ns
SIMPER Similarity (%)[d]	ns	ns	ns

Values presented as means ± standard deviation (±SD); [a]OTUs: Average number of operational taxonomic units; [b]Margalef species richness: d=(S-1)/log(N);[c]Shannons diversity index: H'=-∑(pi(lnpi)); [d]SIMPER, similarity percentage within group replicates; ns: not significant.

However, these results do not agree with the data from other authors which report that microbial communities, including bacteria, differ in different geographical sites, cultivars and climate. These variations in the microbial communities are one of the reasons for the unique characteristics of each wine (Bokulich *et al.*, 2014, Zarraonaindia *et al.*,

2015). Furthermore, other studies have shown changes in the grape surface bacterial community with berry ripening (Renouf *et al.*, 2005). Our divergent results may be due to the limitations of the DGGE technique such as (1) specific primers that do not allow the amplification of all the species; (2) species in low concentration that could not be detected by this method (species detection only above a certain threshold frequency in the population); (3) a ratio of acrylamide that was not optimized may also lead to incorrect separation of the bands in the gel (Prakitchaiwattana *et al.*, 2004, König *et al.*, 2009).

The bands that were excised from the DGGE gel and sequenced originated unclean sequences which impaired the identification of the species. This may be mainly due to an incomplete separation on the bands.

Our culture dependent and independent results are not in agreement as DGGE revealed absence of difference in bacterial community while isolation methodology allow it to identify a huge and diverse community in Alentejo which was not observed in Douro.

4.3. Biogenic amines

4.3.1. Presence of *agdi*, *odc*, *hdc*, *tyrd* and *ldc* genes

The detection of bacteria potentially producers of biogenic amines was achieved through multiplex and uni gene PCR with specific primers for 5 encoding genes – *agdi* and *odc*, *hdc*, *tyrd* and *ldc* – of enzymes responsible for the production of putrescine, histamine, tyrosine and cadaverine, respectively (Fig. 20 and 21). Ninety-seven bacteria were screened. It is important to emphasize that this kind of study reveals only the

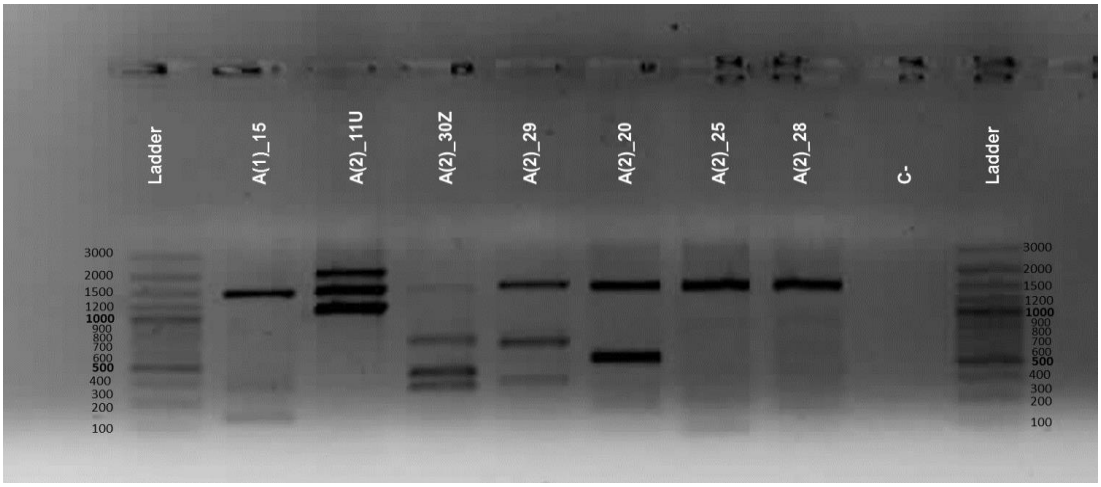


Figure 20 – Electrophoretic agarose gel evidencing the Multiplex PCR products of *agdi*, *tyrd*, *odc* and *hdc* genes amplification from Alentejo isolates. The used Ladder was MR17 DNA Ladder (DNAgdansk); C- stands for Negative Control of the PCR reaction. For isolates designation see Table 4. All the isolates amplified the 16S rRNA gene (band with 1537 bp), used as an internal control of the reaction; A(2)_11U amplified the *tyrd* correspondent band (1133 bp); A(2)_30Z and A(2)_29 amplified the *odc* gene (900 bp) and the *hdc* gene (435 bp) and A(2)_ 20 amplified the *agdi* gene (600 bp).

potential of these bacteria for the production of these enzymes as the real production of the amines cannot be detected by this method.

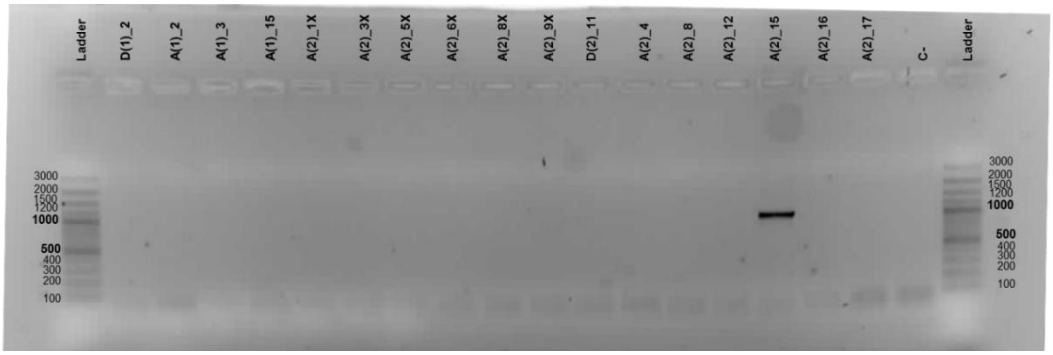


Figure 21 – Electrophoretic agarose gel evidencing the PCR products of *ldc* gene amplification from Douro and Alentejo isolates. The used Ladder was MR17 DNA Ladder (DNAgdansk); C- stands for Negative Control of the PCR reaction. For isolates designation see Table 4. Only A(2)_15 has demonstrated to have the *ldc* gene as seen by the amplification of this gene (1098 bp).

Overall, 55 isolates out of 97 analysed demonstrated the presence of one or more genes related to the production of HDC, ODC, AgDI and TDC enzymes (≈57 %). The most amplified gene in all the screened isolates was *agdi* (n=22), followed by *hdc* (n=20), *odc* (n =19), *tyrd* (n=11) and *ldc* with only one amplification (Fig. 22). In Douro samples, D(1)_# isolate did not demonstrate the presence of any of these genes in its genomic material, while D(2)_# isolates demonstrated the presence of *tyrd* and *hdc* genes. In Alentejo samples, a*gdi* and *hdc*, where detected in A(1)_# bacteria, while A(2) isolates demonstrated the presence of all genes searched. The biogenic amine with a higher probability of being produced by grape bacterial community was putrescine as *agdi* and *odc* amplifications, both responsible for putrescine production, summed up, 56 % (n= 41) of the total amplifications. The remaining 44 % (n=32) are divided by three different

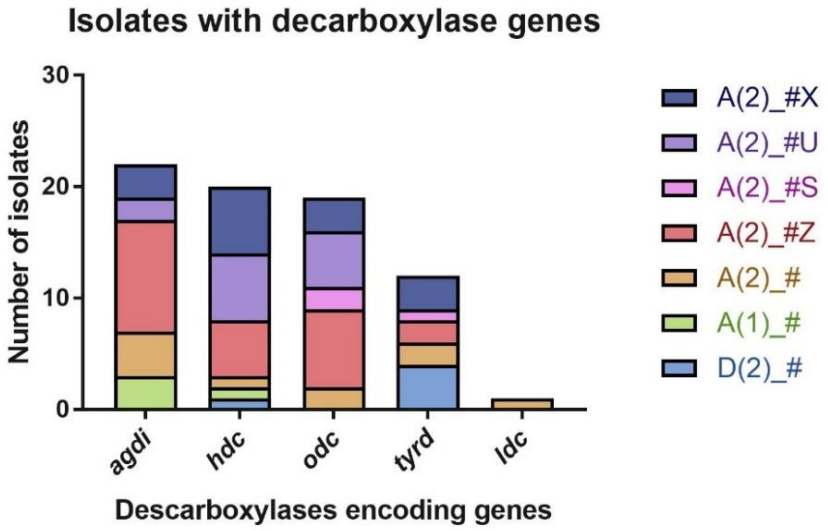


Figure 22 – Number of isolates of each sample that demonstrated the presence of *agdi*, *tyrd*, *odc* and *ldc* genes. 22 isolates demonstrated the presence of *agdi* gene, 20 of *hdc* gene, followed by *odc* presence in 19 isolates, 11 isolates amplified *tyrd* and one amplified *ldc*.

biogenic amine enzymes. Noteworthy is the fact that no isolate from Douro possessed genes related to putrescine production, while in Alentejo (considering just A(1)_# and A(2)_#) the majority of isolates amplified both *agdi* and *odc* genes (Fig. 22). The results obtained revealed that the amines with a higher potential of being produced, by decreasing order, are putrescine, histamine and tyramine. Putrescine has been described as the amine present in higher levels in red wines followed by histamine which was only found in some samples and in lower levels (Spano *et al.*, 2010). In this study the presence of cadaverine was also described but not tyramine. In our study, cadaverine was not found but tyramine was. The majority of the BA^+ isolates did not have more than one gene, with no isolate demonstrating the 4 genes (*agdi*, *tyrd*, *odc* and *hdc*) and just two demonstrating to have three genes (*agdi*, *odc* and *hdc*; *tyrd*, *odc* and *hdc*) (Fig. 23). In a study with LAB

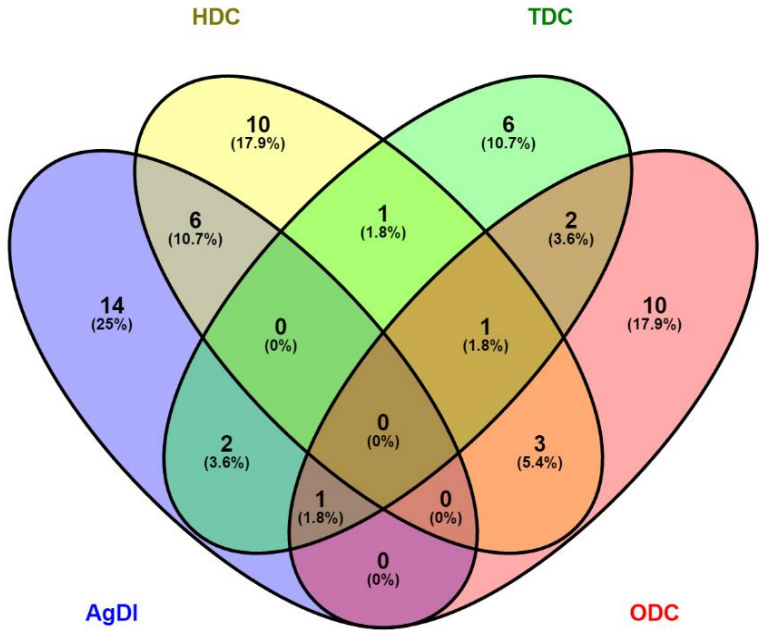


Figure 23 – Number of isolates that have the potential to produce one or more ODC, AgDI, HDC and TDC enzymes, as amplified for the correspondent genes. 52 % of the studied isolates amplified only the *agdi* gene, 17.9 % only the *odc* gene and the same percentage the *hdc* gene while only 6 (10.7 %) amplified the *tyrd* gene. Regarding isolates that amplified three genes only 1.8% amplified for *odc*, *hdc* and *tyrd* and 1.8 % for the *agdi*, *tyrd* and *odc*. Amplification of *agdi* and *odc* was achieved by 3.6% of isolates, 1.8% for the *hdc* and *tyrd*, 10.7 % for *agdi* and *hdc*, 5.4 % for *odc* and *hdc* and 3.6 % for *odc* and *tyrd*. No isolate amplified the four genes

species (from *Lactobacillales* order), some bacterial strains were commonly found to possess the genes *tyrd* and *agdi,* which are two pathways for which genes are thought to be transferred together between strains by horizontal gene transfer and are also thought to be linked (Luca*s et al.,* 2007, Coton & Coton, 2009). In our study, only two isolates demonstrated the presence of both these genes; however, our strains were not LAB strains, which may explain the difference in results. Furthermore, *agdi* was the gene mostly detected (Fig. 22 and 24). There are some contradictory information about these two

genes. In fact, *agdi* has been described as the most commonly present in wine bacteria and *odc* the least found (Coton *et al.*, 2010), which does not totally correspond to the present results. However, there are other studies that state that *odc* is more common in wine and *agdi* in cheeses (Romano *et al.*, 2012). The high number of *odc*$^+$ and *agdi*$^+$ strains may relate to the high values of putrescine in Alentejo grapes.

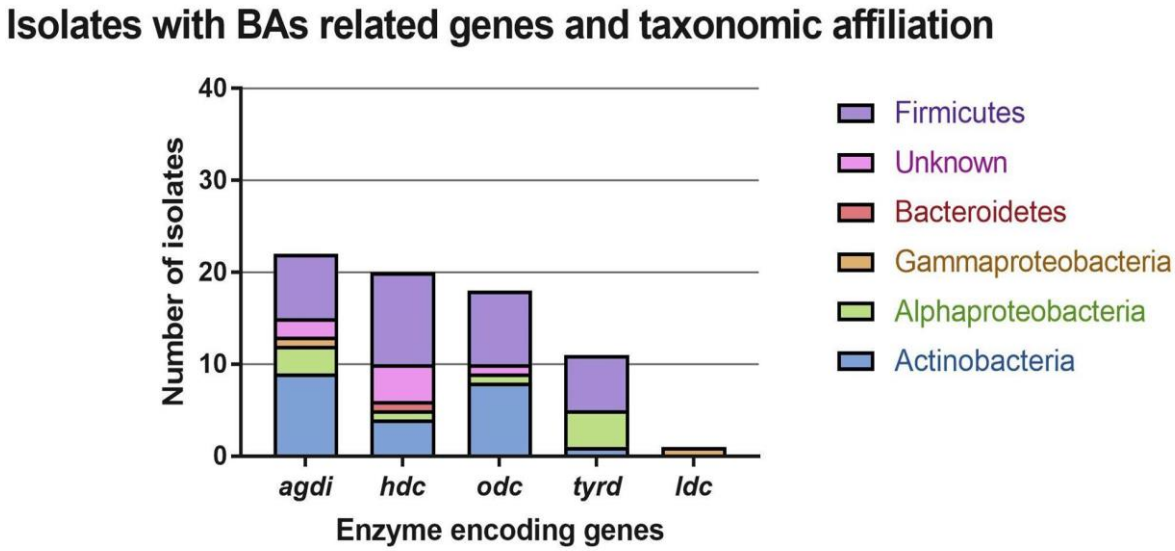


Figure 24 – Number of isolates from each phylum that demonstrated the presence of one or more genes. 22 isolates demonstrated the presence of *agdi* gene (9 *Actinobacteria*, 3 *Alphaproteobacteria*, 1 *Gammaproteobacteria*, 7 *Firmicutes*,2 Unknown), 20 of *hdc* gene gene (4 *Actinobacteria*, 1 *Alphaproteobacteria*, 1 *Bacteroidete*, 10 *Firmicutes*,4 Unknown), followed by *odc* (8 *Actinobacteria*, 1 *Alphaproteobacteria*, 6 *Firmicutes*, 2 Unknown) presence in 18 isolates, 11 isolates amplified *tyrd* (1 *Actinobacteria*, 4 *Alphaproteobacteria*, 6 *Firmicutes*) and one amplified *ldc* (1 *Bacteroidetes*).

Even though *hdc* was the most frequent gene, no histamine was detected in musts or grapes of both wine regions. HDC can be used by bacteria as a mean to obtain additional energy under less favourable conditions, leading to the formation of histamine (Konings *et al.*, 1997). In wine, it has been described that the presence of *hdc* gene does not necessarily implies the production of histamine (Coton *et al.*, 1998), as the presence of substrates, such as glucose and malic acid, makes unnecessary the use of this additional energy pathway (Lonvaud-Funel, 2001, Smit, 2008). However, the high frequency of *hdc*$^+$ bacterial strains is concordant with previous findings (Landete *et al.*, 2005, Nannelli *et al.*, 2008).

Although cadaverine is commonly detected in wine samples, it was only observed one *ldc*$^+$ isolate. Other studies had also encountered a small or inexistent panel of strains with the *ldc* gene and it is hypothesized that the found production of cadaverine may be related to a less specific *odc* pathway which converts lysine into cadaverine (Guirard & Snell, 1980, Guerrini *et al.*, 2001). However, in the current study, cadaverine in musts and grapes was not detected which is in agreement with the results of our gene screening.

Regarding the affiliation of BA$^+$ bacteria, 18 *Actinobacteria* (86%), 8 *Proteobacteria* (1 *Gammaproteobacteria* and 7 *Alphaproteobacteria* (64%)), 21 *Firmicutes* (49%) and 1 *Bacteroidetes* demonstrated the presence of one or more genes (Fig. 24, Annex II). The isolates were distributed by 18 genera with no noticeable pattern of gene distribution (Fig. 25). The only *ldc*$^+$ isolate that is also *agdi*$^+$, belong to a *Gammaproteobacteria*, from *Enterobacter* genus. This genus has been described as a putrescine producing genus (Lavizzari *et al.*, 2010) and *E. cloacae* has been referred as producer of putrescine and cadaverine, in sausages, anchovies and spinaches (Pons-Sanchez-Cascado *et al.*, 2005, Lavizzari *et al.*, 2010, Curiel *et al.*, 2011). No reference exists to BAs production by this species in wine. Five different genera from Firmicutes have isolates that demonstrated the presence of BA genes, being *Bacilllus* the genus with a higher number of isolates (Fig.25). *Pediococcus pentosaceus* was the only LAB isolated in this studied and demonstrated the presence of *hdc* gene. This species has been described as an amine producer in wine, mainly related to the AgDI pathway and, consequently, the putrescine production (Coton *et al.*, 2010). Furthermore, the genus *Pediococcus*, although thought to be present in low proportion, is referred as the genus mostly responsible for the presence of histamine in

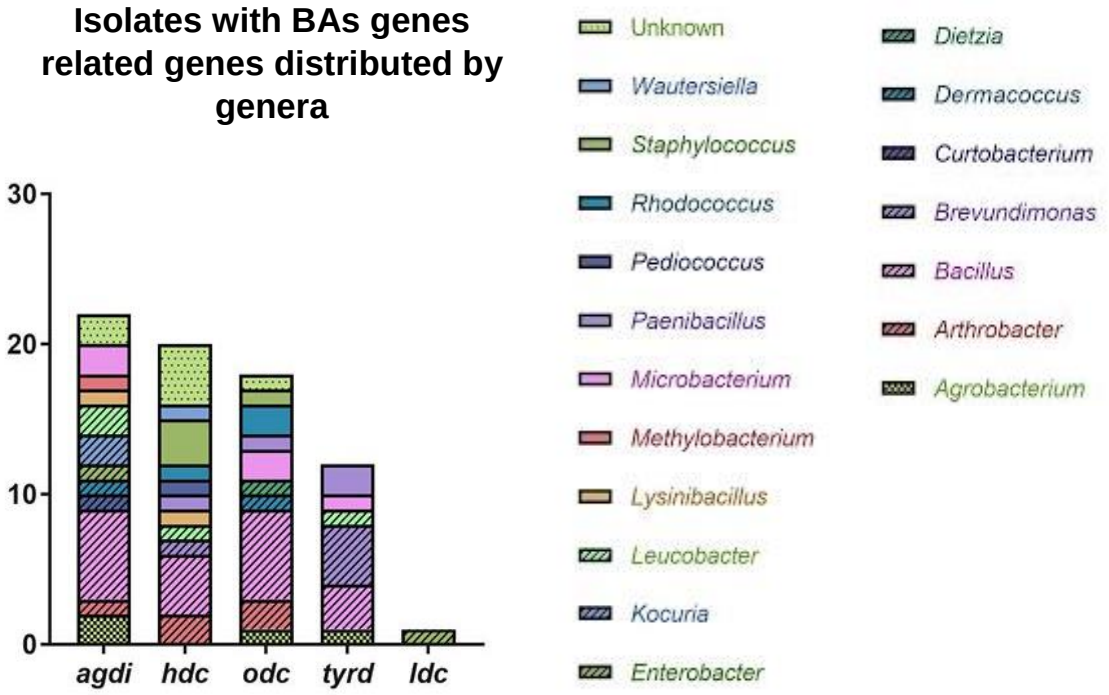


Figure 25 – Number of isolates from each genus that demonstrated the presence of one or more genes. 22 isolates demonstrated the presence of *agdi* gene (2 *Agrobacterium*, 1 *Arthrobacter*, 1 *Curtobacterium*, 1 *Dermacoccus*, 2 *Kocuria*, 2 *Leucobacter*, 1 *Lysinibacillus*, 1 *Methylobacterium*, 2 *Microbacterium*, 2 Unknown), 20 of *hdc* gene gene (2 *Arthrobacter*, 4 *Bacillus*, 1 *Brevundimonas*, 1 *Leucobacter*,1 *Lysinibacillus*, 1 *Paenibacillus*, 1 *Pediococcus*, 1 *Rhodococcus*, 3 *Staphylococcus*, 1 *Wautersiella*, 4 Unknown), followed by *odc* (1 *Agrobacterium*, 2 *Arthrobacter*, 7 *Bacillus*, 1 *Dermacoccus*, 1 *Dietzia*, 2 *Microbacterium*, 1 *Paenibacillus*, 2 *Rhodococcus*, 1 *Staphylococcus*, 1 Unknown) presence in 19 isolates, 11 isolates amplified *tyrd* (1 *Agrobacterium*, 3 *Bacillus*, 4 *Brevundimonas*, 1 *Leucobacter*, 1 *Microbacterium*, 2 *Paenibacillus*) and one amplified *ldc*. (1 *Enterobacter*).

wine (Lonvaud-Funel & Joyeux, 1994, Le Jeune *et al.*, 1995, Coton *et al.*, 1998, Moreno-Arribas *et al.*, 2003, Landete *et al.*, 2005). Even though it is known that this genus possess the *hdc* gene (Landete *et al.*, 2005, Landete *et al.*, 2007), none of these studies referred histamine production by this bacterium (Landete *et al.*, 2007). *Staphylococcus* had two isolates with presence of BAs genes, *Staphylococcus sp. (hdc*$^+$ and *odc*$^+$*)* and *Staphylococcus saprophytes (hdc*$^+$*). Staphylococcus* is often referred in bibliography as a BAs producing genus in food products, as sausages and fish (Martuscelli *et al.*, 2000, Pons-Sanchez-Cascado *et al.*, 2005, Bermudez *et al.*, 2012), but not in wine. In fact, *Staphylococcus sp.* and *S. saprophytes* had been described as potential producers of tyramine, histidine, putrescine and cadaverine (Santos, 1996, Pons-Sanchez-Cascado *et al.*, 2005, Bermudez *et al.*, 2012). More recently, studies regarding this genus and its ability to produce BAs lead to the discovery of an *hdc*$^+$ *Staphylococcus epidermis* strain in grape musts (Benavent-Gil *et al.*, 2016). One isolate affiliated to *Lysinibacillus fusiformis* demonstrated the presence of *hdc* and *agdi* genes. *Lysinibacillus fusiformis* as found to be a producer of histamine, putrescine and cadaverine in cheeses (Pachlová *et al.*, 2016). Fifteen isolates identified as *Bacillus* had also demonstrated the presence of BA encoding genes. *Bacillus* has been described as BAs producing bacteria (Bermudez *et al.*, 2012, Chang & Chang, 2012), although studies about these subjects are not so common, especially in wine. Even so, *Bacillus amylodiquefaciens*, *B. anthracis*, *B. cereus*, *B. pumilus* have previously been described as BAs producers (Hernández-Herrero *et al.*, 1999, Min *et al.*, 2004, Chang *et al.*, 2009, Bermudez *et al.*, 2012, Chang & Chang, 2012, Benkerroum, 2016) and isolates related to these species demonstrated the presence of BAs related genes in our study. Our *Bacillus amylodiquefaciens* isolate is *hdc*$^+$ and *agdi*$^+$, *B. cereus* is *odc*$^+$, *B*. *pumilus* is *hdc*$^+$. Only *B. pumilis* has been described in wine (Chang *et al.*, 2009). Contrarily to our results, where *B. anthracis* demonstrated to be *odc*$^+$ this species has only been referred spermidine producer (Benkerroum, 2016). *Paenibacillus*, although described as present in vine soil (Martins *et al.*, 2013), it has not been referred in any study related to biogenic amines production. In our study, we found two Paenibacillus graminis strains *tyrd*$^+$ and *hdc*$^+$/*tyrd*$^+$/*odc*$^+$.

Although we found 18 Actinobacterial isolates with BAs related genes, the majority of the studies regarding this phylum reports its capacity for degrading these molecules, through the presence of amine oxidases (Martuscelli *et al.*, 2000, Naila *et al.*, 2010, Callejón *et al.*, 2015), and not for its synthesis. As an exception, a different strain of *Kocuria* has been described to produce histamine, which differs from our *agdi*$^+$ *Kocuria* strains*.* Thus, our results regarding BAs production potential in *Actinobacteria* was unexpected and not supported by the literature. Likewise, the results from the 5 *Alphaproteobacteria* isolated in this dissertation (2 *Agrobacterium tumefaciens* – *agdi*$^+$ and *agdi*$^+$, *odc*$^+$ and

tyrd^{+}- and 3 *Brevundimonas* (2 *Brevundimonas* sp. and *Brevundimonas diminuta* – *tyrd*$^{+}$)) have also no bibliographic support. The *hdc*$^{+}$ *Wautersiella falsenii* will not be discussed as it has been considered as a contaminant.

4.3.2. BA production by pure cultures of bacterial isolates after growth in decarboxylase media

After the screening of the potential of the bacteria to produce the BAs, through the detection of the genes, the isolates were studied for their real capacity to produce the respective BA. Thus, isolates that demonstrated the presence of BAs were cultivated in a specific decarboxylase medium supplemented with the BA amino acid precursor and a pH indicator (Fig.26).

After the incubation period, the specific culture medium would change colour becoming yellow in more basic (indicative of BAs production) (Fig. 26C) or purple if more acidic (indicative of no BAs production) (Fig. 26A). Although this do not happen in all isolates, all the culture media was sent to analysis by LC-MS technique. Due to technical difficulties, it was not possible to obtain results of these analyses. In the samples analysed no increment in levels of BAs was obtained comparatively to the control, incubated medium without bacteria, that has high levels of BAs.

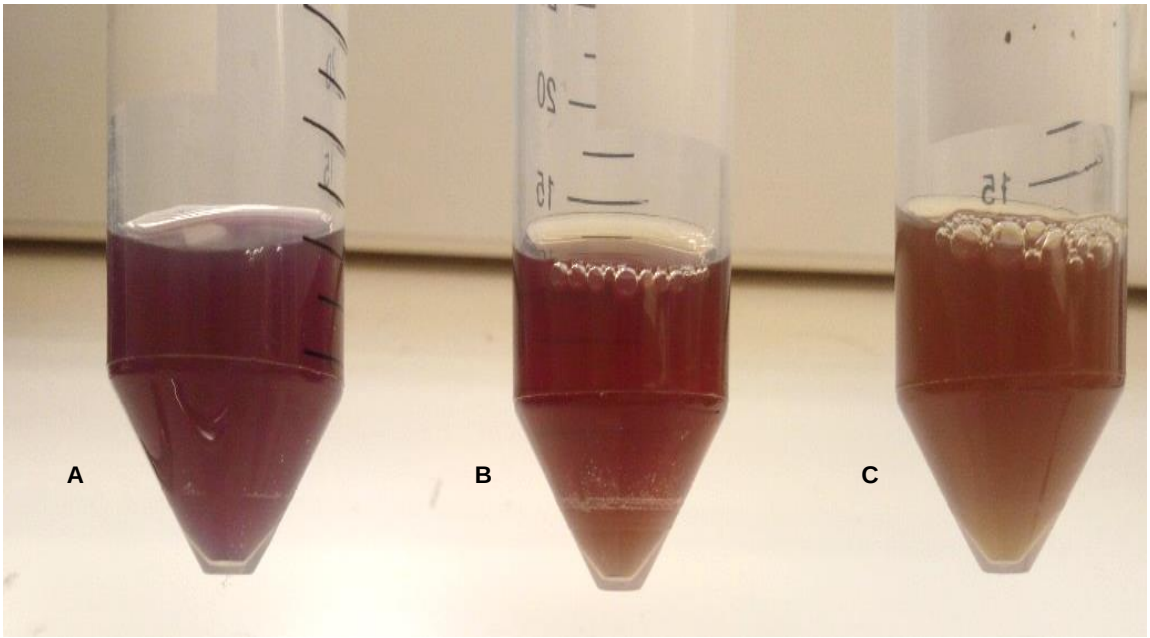


Figure 26 – Example of the colours obtained after incubation of isolates in decarboxylase media. A - Media after acidification. B - Initial colour of the medium. C- media after alkalinisation.

4.4. Bioactive potential of the isolates

The bioactive potential of the bacterial isolates was screened because antimicrobial activity by some bacteria was detected during isolation (Fig. 27). Only 97 isolates were analysed regarding their bioactive potential through the analysis of the presence of NRPS and PKS-I genes in their genomes. The products of the PCR reaction were visualized by gel electrophoresis for the presence of approximately 700 bp and 1000

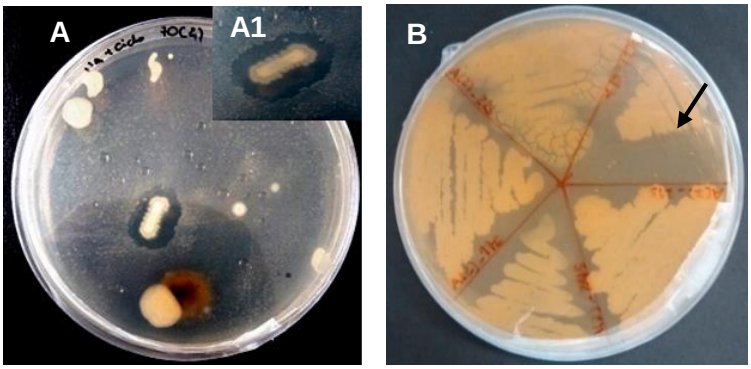


Figure 27 – Isolates that demonstrated antimicrobial activity during isolation. A – During the spreading of the extracts. A1 is a zoom in the area of interest. B- During the isolation procedures. The arrow indicates the area of interest.

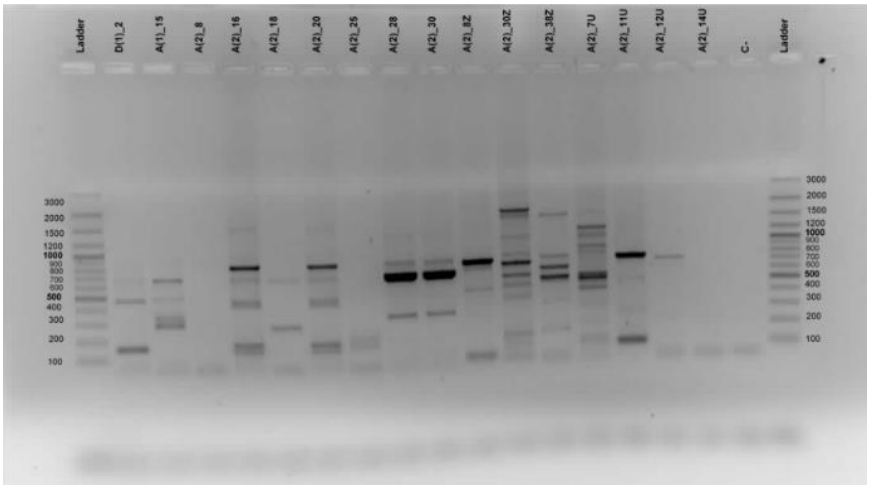


Figure 28 – Electrophoretic agarose gel evidencing the PCR products of *PKS-I* gene amplification from Alentejo and Douro isolates. The used Ladder was MR17 DNA Ladder (DNAgdansk); C- stands for Negative Control of the PCR reaction. For isolates designation see Table 4. *PKS-I* amplifies 700 bp.

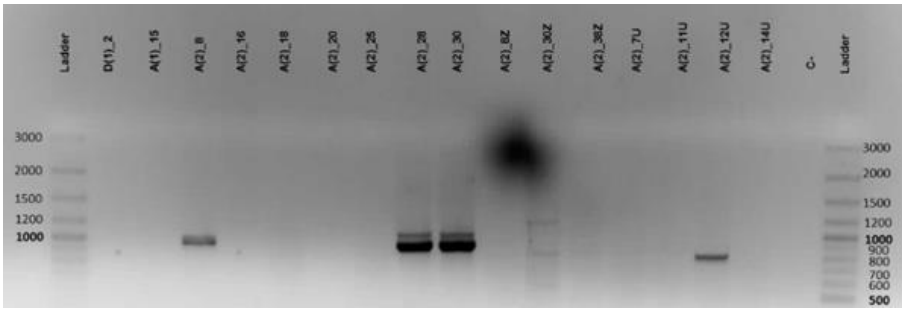


Figure 29 – Electrophoretic agarose gel evidencing the PCR products of *NRPS* gene amplification from Alentejo and Douro isolates. The used Ladder was MR17 DNA Ladder (DNAgdansk); C- stands for Negative Control of the PCR reaction. For isolates designation see Table 4. *NRPS* amplifies 1000 bp.

bp size amplicons (Fig. 28 and 29) respectively for PKS-I and NRPS genes (Neilan *et al.*,1999; Kim *et al*., 2005). PKS-I and NRPS genes are gene clusters responsible for the codification of polyketide synthases and nonribosomal peptides synthetases, respectively (Grozdanov & Hentschel, 2007). These enzymes are responsible for the synthesis of many different secondary metabolites that can have relevance as antibiotics or/and antifungals (Hutchinson, 2003, Ansari *et al.*, 2004, Kennedy *et al.*, 2007).

No gene amplification of the two genes was observed in Douro bacteria (Fig. 30). On the other hand, in Alentejo 22 isolates were positive for PKS-I and 7 for NRPS (Fig. 30, Annex II). Higher number of PKS-I comparatively to NRPS genes is a general trend observed in different studies (Graca *et al.*, 2013, Graça *et al.*, 2016). PKS-I and NRPS positive isolates were affiliated to *Firmicutes*, *Actinobacteria* and *Proteobacteria,* which are phyla known to possess these gene clusters and to be the three groups more abundant in these genes (Kennedy *et al.*, 2009, Miller *et al.*, 2012, Wang *et al.*, 2014) (Fig.31). Values of 25%-50% of bioactive strains were observed for these phyla.

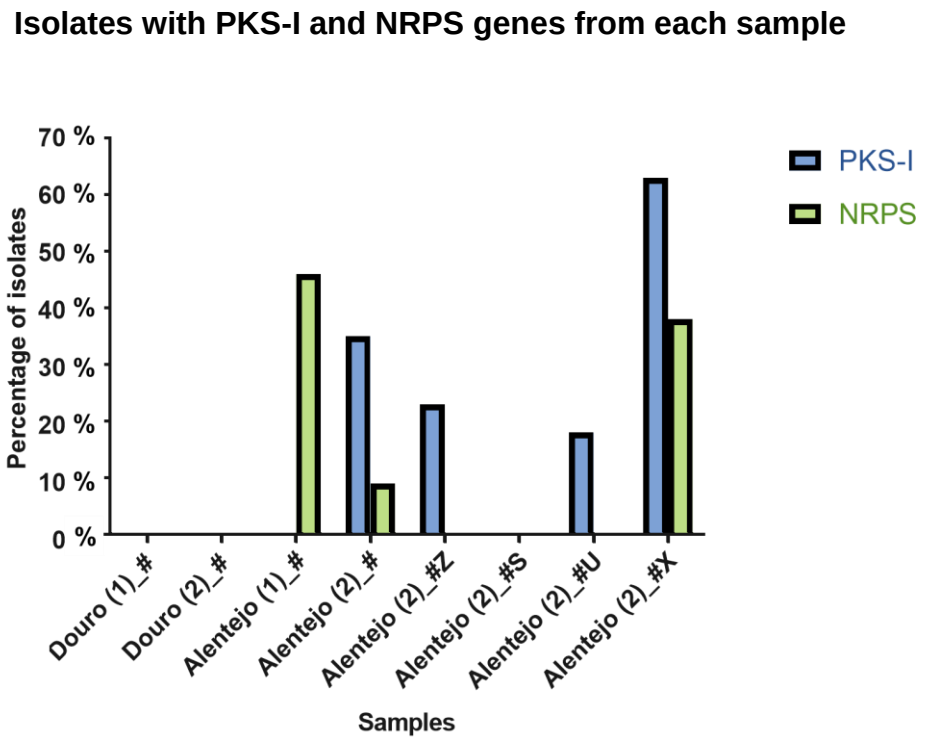


Figure 30 – Percentage of isolates from each sample that demonstrated the presence of *NRPS* and *PKS-I* genes. No isolate from D(1)_#, D(2)_# and A(2)_#S demonstrated the presence of any of the genes. A(1)_# isolates only demonstrated the presence of NRPS genes (46%, n= 5), 35% of A(2)_# isolates possess PKS-I (n=8) and 9% possess NRPS gene (n=2), A(2)_#Z and A(2)_#U isolates only demonstrated the presence of PKS-I (23%, n=7 and 18%,n=2, respectively) and 63% (n=7) and 38% (n=4) of A(2)_#X possess PKS-I and NRPS genes, respectively.

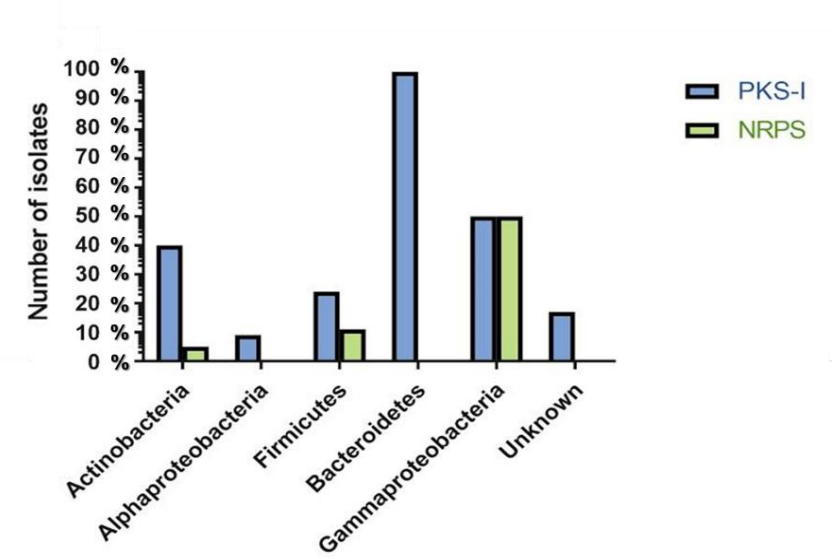


Figure 31 – Percentage of isolates that demonstrated to possess *NRPS* or *PKS-I* genes.

Figure 32 shows the different positive genera regarding PKS-I and NRPS. *Bacillus* and *Paenibacillus* are genera known to possess PKS-I and NRPS genes (Chen *et al.*, 2006, Butcher *et al.*, 2007, Arguelles-Arias *et al.*, 2009, Miller *et al.*, 2012, Graca *et al.*, 2013, Aleti *et al.*, 2015) as well as *Rhodococcus* (Doroghazi & Metcalf, 2013, Komaki *et al.*, 2014, Müller *et al.*, 2015). *Bacillus amyloliquefaciens* (as isolate A(2)_3X) has even been referred to have potential to fight plant plagues through the products resulting from NRPS and PKS gene clusters *(*Arguelles-Arias *et al.,* 2009). Our isolate A(2)_30 had a PKS-I and a NRPS genes and is affiliated to *Paenibacillus polymyxa* strain RCP6 that

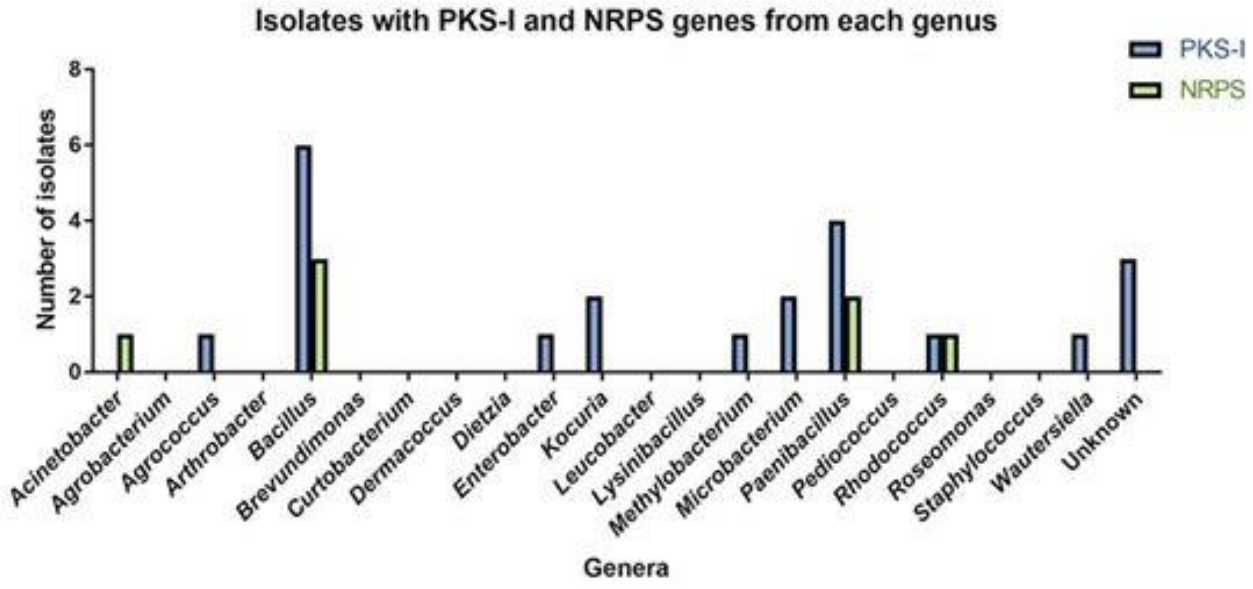


Figure 32 – Number of isolates that demonstrated to possess *NRPS* or *PKS-I* genes, from each genus.

demonstrated strong antagonistic activity against several fungal phytopathogens (Webpage 1). *Actinobacter* has already being described as possessing NRPS clusters (Allen & Gulick, 2014, Wang *et al.*, 2014) and has been bioactive against other bacteria (Graca *et al.*, 2013). *Kocuria* is known to possess PKS genes in its genome (Palomo *et al.*, 2013) as well as *Enterobacter* (Engel *et al.*, 2015).

PKS-I genes were also found in the *Actinobacteria*: *Micrococcus* and *Agrococcus*, and in the *Proteobacteria*: *Methylobacterium* (Annex II), for which no bioactive potential has been referred in the literature.

These data revealed that the Alentejo grape bacterial community possesses a considerable potential to compete in their environment between themselves or fight against organisms like fungi. Regarding Douro as a low number of isolates were obtained and from only two genera (*Brevundimonas* and *Curtobacterium*) no conclusions can be taken regarding the bioactive capacity of Douro grape bacterial community.

4.5. Scanning Electron Microscopy

The presence of bacteria or other biological forms is very scarce and uneven on the surface of grapes (Fig. 33), especially in Douro samples as expected due to our isolation results. In Alentejo mature grapes, bacteria were very difficult to visualize, due to

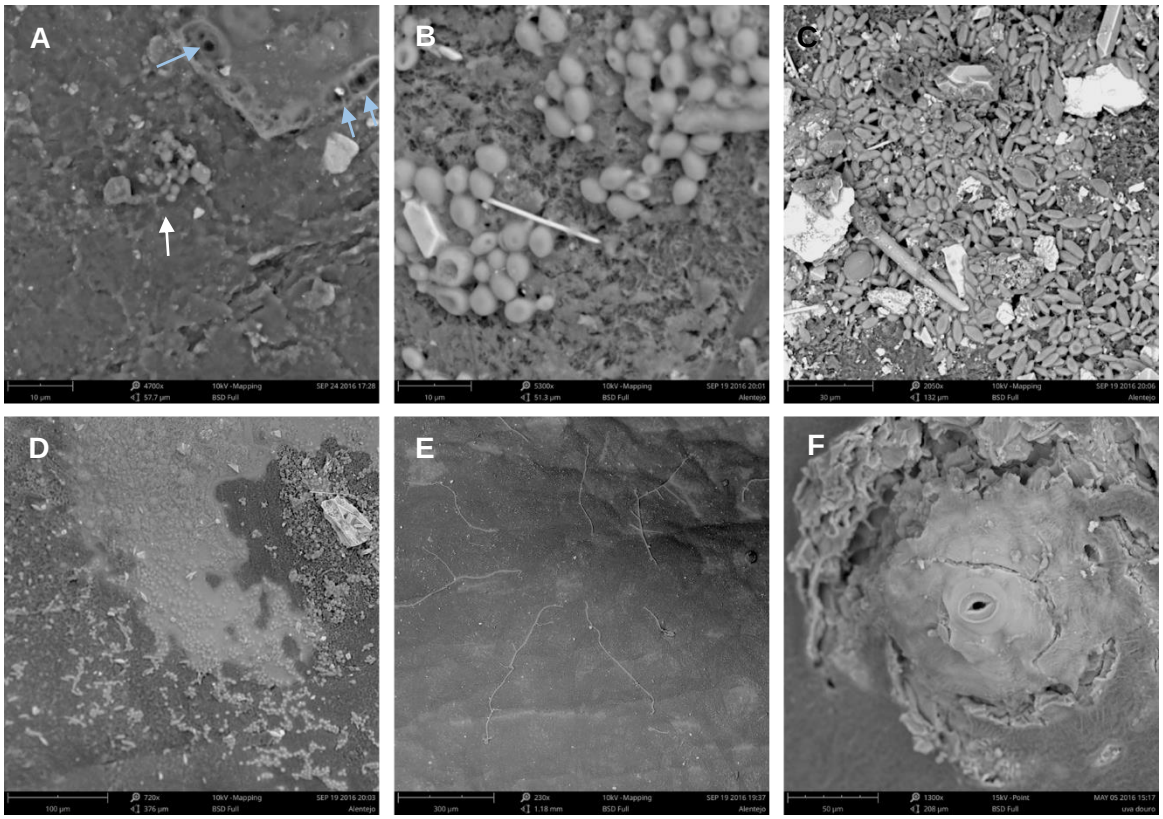


Figure 33 – Images of grape surfaces, showing A - potential bacteria (white arrow) and pore-like structures (blue arrows); B – budding yeasts; C – a complex biofilm of different sizes microbiome; D – mucilaginous material containing mainly yeasts; E – hyphal fungi and F – a stoma in grape surface from Douro**.**

their presumed reduce size (Fig.33A), while yeasts and filamentous fungi were clearly seen (Fig. 33B and E). Frequently, areas with mucilaginous-like material were present, which was altered by the heat of the electron beam. Under this material a great number of yeasts, some under budding, were found (Fig 33D). In Figure 33C, a complex biofilm of cells of different size and shape can be observed. We also observed stomata in Douro grapes (Fig.33F) and other pore-like structures that are surrounded by dense material (Fig. 33A).

Typically, the surface of the grape is a nutritional poor environment that changes with grape ripening because of exudation (Padgett & Morrison, 1990). As already referred, we verified the presence of this material associated to the microorganisms. However, we can hypothesize that bacteria are associated with this material, making it difficult for bacterial observation. Exudation may occur through the stomata or the pore-like structures that we frequently observed on the grapes surface.

4.6. Overall discussion

The main objective of this dissertation was to try to get a better understanding of the reason why Alentejo musts have higher levels of BAs comparatively to the Douro ones, in spite of the similar growth conditions of the grape vines.

An initial hypothesis that has been raised to justify this difference was related to a metabolic impairment of the grape vines due to potassium deficiency in Alentejo. It is known that potassium deficiency in soils induces the production of BAs in plants (Konings *et al.*, 1997). However, this hypothesis was discarded because the levels of potassium in Alentejo soils were higher than in Douro. A potential bacterial biogenic hypothesis for the high BAs levels is thus plausible and need confirmation. The results of this dissertation contributes to in depth this bacterial hypothesis.

A first striking difference between the two regions was the number of isolates obtained. This was much lower in Douro comparatively to Alentejo. Furthermore, the grape bacterial community in Alentejo was much more diverse than that of Douro.

The chemical BAs analyses of grapes and musts confirmed the trend obtained in previous years: higher BAs in Alentejo comparatively to Douro. It also allowed to realise that the levels of BAs increase considerably between the mature grapes and after their maceration in the initial phase of musts. This was very evident in Alentejo where the putrescine levels raised from 1.244 mg/L to 15.736 mg/L.

Furthermore, the analysis of the genetic potential of Alentejo bacteria for BAs production, namely putrescine, was very high. (41 putrescine positive bacteria (*odc*+ and *agdi*$^{+}$)).

The initial notorious increase of BAs in musts should have their origin in the bacterial community present in grapes presenting high biogenic amine potential.

High bacterial number and diversity associated to a high biogenic amine potential may justify the sudden increase of BAs levels in musts after maceration of the grapes, while the pH was still less acidic as it is in wine. During the initial phase of wine preparation, bacterial community present in grapes is suddenly exposed to very favourable growth conditions (nutrients, temperature and pH). Furthermore, in Alentejo, the grapes possess a higher number and diverse bacterial community that revealed great potential for putrescine production. With this combination of factors, the levels of this BA has the conditions to increase quickly. As musts are posteriorly acidified with tartaric acid, the initial better conditions for the growth of the bacterial grape community become less favourable, and, in fact, after one week the levels of BAs in Alentejo musts did not increase, as could be expected. The control of BAs production in musts may be due to its low pH and to the production of alcohol during fermentation processes in wine which can control the BA producing bacteria from the grapes (Ancín-Azpilicueta *et al*., 2008).

Although the aim of this study was not to assess the role of yeasts in this process, we can assure that the mature grapes in Alentejo possess high concentration of yeasts on their surface as this were observed by SEM. Furthermore, the yeasts observed were in the process of budding.

5. Conclusions

Grapes and grapevine environments are inhabited by a diverse community of microorganisms which play a major role in wine production but can also produce undesirable molecules. Biogenic amines are one of these compounds. High levels of BAs were detected in musts from a grape vineyard from Alentejo.

Although some studies have been performed relating BAs in wine and bacteria, few have been done regarding the whole bacterial community and, to our knowledge, none has been done in these two regions.

Confirmation of a microbial biofilm in the grapes was obtained by scanning electron microscopy. As observed by a culture-dependent technique, Alentejo grapes revealed to have a higher and more diverse bacterial community than Douro grapes. *Firmicutes* was the dominant group, but *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidete* were also found. Our culture-dependent study points out to a strong influence of biogeography on the bacterial community of the grapes. However, DGGE grape bacterial community profiles did not reveal these differences. In fact, this culture-independent methodology showed a great similarity between bacterial profiles of different grape maturation stages and musts and also between regions. Regarding BAs potential production, bacteria from Alentejo possessed a much higher potential for the production of these amines, especially putrescine, as they revealed a high number of genes in their genome encoding for the enzymes responsible for this molecule. Higher bacterial number and diversity associated to a high BAs potential production in Alentejo comparatively to Douro may explain the high levels of BAs in Alentejo musts. Although *Actinobacteria* are normally considered BAs degrading organisms, in fact we observed a great percentage of BAs producing bacteria.

Additionally, a molecular analysis of secondary metabolite genes evidenced the great bioactive potential of bacteria isolated from grapes. The complexity present in grape communities may have favoured species with high bioactive potential. These bacteria may contribute to supress the growth of the disease-causing pathogens on grapes.

6. Future perspectives

The work presented in this dissertation is a relevant contribution to the study of the bacterial diversity found on grapes and their role in BAs levels in musts. The complete obtainment of the data of this study proved to be difficult in a one-year time due to the time consuming of some of the studies. Therefore, the next steps should be the conclusion for all the bacterial isolates of some topics such as their identification and the assessment of the presence of BAs and PKS-I and NRPS genes.

As the samples are only available once a year, confirmation of some results could not be done. This is the case of the DGGE analysis of bacterial communities between Douro and Alentejo samples for which confirmation of the unexpected results should be done. The improvement of the DGGE technique as well as sequencing of the obtained bands would allow additional information. An attempt to extract bacterial DNA from each grape part (skin and pulp) would also permit to confirm the localization of the community within the grape, as no isolate was retrieved from the interior of the grape.

Further optimization of the growth experiments for the production of BAs and their chemical quantification should be attempted as well as assayed a different method described by Chang & Chang (2008). Also, qPCR technique would allow to assess gene transcription, giving us a better picture of the real capacity of the bacteria for the production of BAs.

The bacterial community of grapes has revealed a potential to produce bioactive compounds and, thus, it is important to study in detail this potential and, for example, perform bioactivity assays and analysis of other genes known to be involved in the production of secondary metabolites.

An important study to assessed more in depth the bacterial communities of Douro and Alentejo should be performed by metagenomics analysis of the bacterial community, by Next-Generation Sequencing.

The publication of the manuscript in a peer review journal is envisaged.

7. References

Abe K, Ohnishi F, Yagi K, Nakajima T, Higuchi T, Sano M, Machida M, Sarker RI & Maloney PC (2002) Plasmid-Encoded asp Operon Confers a Proton Motive Metabolic Cycle Catalyzed by an Aspartate-Alanine Exchange Reaction. *Journal of Bacteriology* **184**: 2906-2913.

Agudelo-Romero P, Bortolloti C, Pais MS, Tiburcio AF & Fortes AM (2013) Study of polyamines during grape ripening indicate an important role of polyamine catabolism. *Plant Physiology and Biochemistry* **67**: 105-119.

Aleti G, Sessitsch A & Brader G (2015) Genome mining: Prediction of lipopeptides and polyketides from *Bacillus* and related *Firmicutes*. *Computational and Structural Biotechnology Journal* **13**: 192-203.

Allen CL & Gulick AM (2014) Structural and bioinformatic characterization of an *Acinetobacter baumannii* type II carrier protein. *Acta crystallographica Section D, Biological crystallography* **70**: 1718-1725.

Ancín-Azpilicueta C, González-Marco A & Jiménez-Moreno N (2008) Current Knowledge about the Presence of Amines in Wine. *Critical Reviews in Food Science and Nutrition* **48**: 257-275.

Anlı RE & Bayram M (2008) Biogenic Amines in Wines. *Food Reviews International* **25**: 86-102.

Anlı RE, Vural N, Yılmaz S & Vural ŸH (2004) The determination of biogenic amines in Turkish red wines. *Journal of Food Composition and Analysis* **17**: 53-62.

Ansari MZ, Yadav G, Gokhale RS & Mohanty D (2004) NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. *Nucleic Acids Research* **32**: W405-W413.

Arena ME & Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. *Journal of Applied Microbiology* **90**: 158-162.

Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Joris B & Fickers P (2009) *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. *Microbial Cell Factories* **8**: 1-12.

Bae S, Fleet GH & Heard GM (2006) Lactic acid bacteria associated with wine grapes from several Australian vineyards. *Journal of Applied Microbiology* **100**: 712-727.

Baldan E, Nigris S, Populin F, Zottini M, Squartini A & Baldan B (2014) Identification of culturable bacterial endophyte community isolated from tissues of *Vitis vinifera* "Glera". *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* **148**: 508-516.

Barata A, Malfeito-Ferreira M Fau - Loureiro V & Loureiro V (2012) The microbial ecology of wine grape berries.

Barbe JC, De Revel G, Joyeux A, Bertrand A & Lonvaud-Funel A (2001) Role of botrytized grape micro-organisms in SO_2 binding phenomena. *Journal of Applied Microbiology* **90**: 34-42.

Bardócz S, Grant G, Brown D, Ralph A & Pusztai A (1993) Polyamines in food—implications for growth and health. *The Journal of Nutritional Biochemistry* **4**: 66-71.

Benavent-Gil Y, Berbegal C, Lucio O, Pardo I & Ferrer S (2016) A new fear in wine: Isolation of *Staphylococcus epidermidis* histamine producer. *Food Control* **62**: 142-149.

Benkerroum N (2016) Biogenic Amines in Dairy Products: Origin, Incidence, and Control Means. *Comprehensive Reviews in Food Science and Food Safety* **15**: 801-826.

Bermudez R, Lorenzo JM, Fonseca S, Franco I & Carballo J (2012) Strains of *Staphylococcus* and *Bacillus* isolated from traditional sausages as producers of biogenic amines. *Front Microbiol* **3**: 151.

Bokulich NA, Thorngate JH, Richardson PM & Mills DA (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences* **111**: E139-E148.

Bray JR & Curtis JT (1957) An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs* **27**: 325-349.

Butcher RA, Schroeder FC, Fischbach MA, Straight PD, Kolter R, Walsh CT & Clardy J (2007) The identification of bacillaene, the product of the PksX megacomplex in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences* **104**: 1506-1509.

Callejón S, Sendra R, Ferrer S & Pardo I (2015) Ability of *Kocuria varians* LTH 1540 To Degrade Putrescine: Identification and Characterization of a Novel Amine Oxidase. *Journal of Agricultural and Food Chemistry* **63**: 4170-4178.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN & Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research* **16**: 11141-11156.

Chang M & Chang HC (2012) Development of a screening method for biogenic amine producing *Bacillus spp. Int J Food Microbiol* **153**: 269-274.

Chang S-C, Lin C-W, Jiang C-M, Chen H-C, Shih M-K, Chen Y-Y & Tsai Y (2009) Histamine production by bacilli bacteria, acetic bacteria and yeast isolated from fruit wines. *LWT - Food Science and Technology* **42**: 280-285.

Charters S (2006) *Wine and Society: The Social and Cultural Context of a Drink*. Elsevier Butterwoth-Heinemann, Oxford.

Chen X-H, Vater J, Piel J*, et al.* (2006) Structural and Functional Characterization of Three Polyketide Synthase Gene Clusters in *Bacillus amyloliquefaciens* FZB 42. *Journal of Bacteriology* **188**: 4024-4036.

Christensen JE, Dudley EG, Pederson JA & Steele JL (1999) Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **76**: 217-246.

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* **18**: 117-143.

Cocolin L, Pepe V, Comitini F, Comi G & Ciani M (2004) Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. *FEMS Yeast Res* **5**: 237-245.

Compant S, Mitter B, Colli-Mull JG, Gangl H & Sessitsch A (2011) Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb Ecol* **62**: 188-197.

Coombe BG (1992) Research on Development and Ripening of the Grape Berry. *American Journal of Enology and Viticulture* **43**: 101-110.

Coton E & Coton M (2009) Evidence of horizontal transfer as origin of strain to strain variation of the tyramine production trait in *Lactobacillus brevis*. *Food Microbiol* **26**: 52-57.

Coton E, Rollan GC & Lonvaud-Funel A (1998) Histidine carboxylase of *Leuconostoc oenos* 9204: purification, kinetic properties, cloning and nucleotide sequence of the *hdc* gene. *J Appl Microbiol* **84**: 143-151.

Coton E, Rollan G, Bertrand A & Lonvaud-Funel A (1998) Histamine-Producing Lactic Acid Bacteria in Wines: Early Detection, Frequency, and Distribution. *American Journal of Enology and Viticulture* **49**: 199.

Coton M, Romano A, Spano G, Ziegler K, Vetrana C, Desmarais C, Lonvaud-Funel A, Lucas P & Coton E (2010) Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. *Food Microbiology* **27**: 1078-1085.

Cunin R, Glansdorff N, Pierard A & Stalon V (1986) Biosynthesis and metabolism of arginine in bacteria. *Microbiological reviews* **50**: 314-352.

Curiel JA, Ruiz-Capillas C, de Las Rivas B, Carrascosa AV, Jimenez-Colmenero F & Munoz R (2011) Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration. *Meat science* **88**: 368-373.

De Man JC, Rogosa M & Sharpe ME (1960) A medium for the cultivation of *Lactobacilli*. *Journal of Applied Bacteriology* **23**: 130-135.

De Vero L, Gala E, Gullo M, Solieri L, Landi S & Giudici P (2006) Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. *Food Microbiol* **23**: 809-813.

Del Prete V, Costantini A, Cecchini F, Morassut M & Garcia-Moruno E (2009) Occurrence of biogenic amines in wine: The role of grapes. *Food Chemistry* **112**: 474-481.

Di Luccia A, Picariello G, Iacomino G, Formisano A, Paduano L & D'Agostino L (2009) The *in vitro* nuclear aggregates of polyamines. *FEBS Journal* **276**: 2324-2335.

Doroghazi JR & Metcalf WW (2013) Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC Genomics* **14**: 1-13.

Dourado MN, Bogas AC, Pomini AM, Andreote FD, Quecine MC, Marsaioli AJ & Araujo WL (2013) *Methylobacterium*-plant interaction genes regulated by plant exudate and quorum sensing molecules. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]* **44**: 1331-1339.

Elnifro EM, Ashshi AM, Cooper RJ & Klapper PE (2000) Multiplex PCR: Optimization and Application in Diagnostic Virology. *Clinical Microbiology Reviews* **13**: 559-570.

Emborg J & Dalgaard P (2008) Modelling the effect of temperature, carbon dioxide, water activity and pH on growth and histamine formation by *Morganella psychrotolerans*. *International Journal of Food Microbiology* **128**: 226-233.

Emmanuel PJ (1993) Polymerase chain reaction from bench to bedside. Applications for infectious disease. *The Journal of the Florida Medical Association* **80**: 627-630.

Engel P, Vizcaino MI & Crawford JM (2015) Gut Symbionts from Distinct Hosts Exhibit Genotoxic Activity via Divergent Colibactin Biosynthesis Pathways. *Applied and Environmental Microbiology* **81**: 1502-1512.

Estreicher SK (2013) Wine. *The Encyclopedia of Ancient History,*p.^pp. John Wiley & Sons, Inc.

Faist H, Keller A, Hentschel U & Deeken R (2016) Grapevine (*Vitis vinifera*) Crown Galls Host Distinct Microbiota. *Applied and Environmental Microbiology* **82**: 5542-5552.

Fernandez M, Linares DM, Rodriguez A & Alvarez MA (2007) Factors affecting tyramine production in *Enterococcus durans* IPLA 655. *Applied Microbiology and Biotechnology* **73**: 1400-1406.

Fernandez M, Florez AB, Linares DM, Mayo B & Alvarez MA (2006) Early PCR detection of tyramine-producing bacteria during cheese production. *Journal of Dairy Research* **73**: 318-321.

Fernández M, del Río B, Linares DM, Martín MC & Alvarez MA (2006) Real-Time Polymerase Chain Reaction for Quantitative Detection of Histamine-Producing Bacteria: Use in Cheese Production. *Journal of Dairy Science* **89**: 3763-3769.

Francesca N, Settanni L, Sannino C, Aponte M & Moschetti G (2011) Ecology and technological capability of lactic acid bacteria isolated during Grillo grape vinification in the Marsala production area. *Annals of Microbiology* **61**: 79-84.

Garijo P, Santamaría P, López R, Sanz S, Olarte C & Gutiérrez AR (2008) The occurrence of fungi, yeasts and bacteria in the air of a Spanish winery during vintage. *International Journal of Food Microbiology* **125**: 141-145.

Gevers D, Huys G & Swings J (2003) *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. *FEMS Microbiology Letters* **225**: 125-130.

Gilbert JA, van der Lelie D & Zarraonaindia I (2014) Microbial terroir for wine grapes. *Proceedings of the National Academy of Sciences* **111**: 5-6.

Giordano C, Falleni M, Capria A-L, Caracciolo F, Petrini M & Barnini S (2016) First report of *Wautersiella falsenii* genomovar 2 isolated from the respiratory tract of an immunosuppressed man. *IDCases* **4**: 27-29.

Goodstadt L & Ponting CP (2001) CHROMA: consensus-based colouring of multiple alignments for publication. *Bioinformatics* **17**: 845-846.

Graca AP, Bondoso J, Gaspar H, Xavier JR, Monteiro MC, de la Cruz M, Oves-Costales D, Vicente F & Lage OM (2013) Antimicrobial Activity of Heterotrophic Bacterial Communities from the Marine Sponge Erylus discophorus (Astrophorida, Geodiidae). *Plos One* **8**.

Graça AP, Calisto R & Lage O (2016) Planctomycetes as Novel Source of Bioactive Molecules. *Frontiers in Microbiology* **7**.

Grozdanov L & Hentschel U (2007) An environmental genomics perspective on the diversity and function of marine sponge-associated microbiota. *Current Opinion in Microbiology* **10**: 215-220.

Guerrini S, Mangani S, Granchi L & Vincenzini M (2001) Biogenic Amine Production by *Oenococcus oeni*. *Current Microbiology* **44**: 374-378.

Guirard BM & Snell EE (1980) Purification and properties of ornithine decarboxylase from *Lactobacillus sp.* 30a. *Journal of Biological Chemistry* **255**: 5960-5964.

Harrison TJ (1998) The polymerase chain reaction--a time of transition from research to routine. *Journal of clinical pathology* **51**: 491-492.

He M, Li X, Liu H, Miller SJ, Wang G & Rensing C (2011) Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain Lysinibacillus fusiformis ZC1. *Journal of hazardous materials* **185**: 682-688.

Helinck S, Perello M-C, Deetae P, de Revel G & Spinnler H-E (2013) Debaryomyces hansenii, Proteus vulgaris, Psychrobacter sp. and Microbacterium foliorum are able to produce biogenic amines. *Dairy Science & Technology* **93**: 191-200.

Hernández-Herrero MM, Roig-Sagués AX, López SEI, Rodríguez-Jerez JJ & Mora-Ventura MT (1999) Total Volatile Basic Nitrogen and other Physico-chemical and Microbiological Characteristics as Related to Ripening of Salted Anchovies. *Journal of Food Science* **64**: 344-347.

Hernández-Jover T, Izquierdo-Pulido M, Veciana-Nogués MT, Mariné-Font A & Vidal-Carou MC (1997) Biogenic Amine and Polyamine Contents in Meat and Meat Products. *Journal of Agricultural and Food Chemistry* **45**: 2098-2102.

Heuer H, Smalla K, van Elsas JD, Trevors JT & Wellington EM (1997) Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. *Modern soil microbiology* 353-373.

Hiratsuka S, Onodera H, Kawai Y, Kubo T, Itoh H & Wada R (2001) ABA and sugar effects on anthocyanin formation in grape berry cultured *in vitro*. *Scientia Horticulturae* **90**: 121-130.

Hou M, Lin S, Yuann ZP, Lin W, Wang AH-J & Kan L-S (2001) Effects of polyamines on the thermal stability and formation kinetics of DNA duplexes with abnormal structure. *Nucleic Acids Research* **29**: 5121-5128.

Hutchinson CR (2003) Polyketide and non-ribosomal peptide synthases: Falling together by coming apart. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 3010-3012.

Inês A, Tenreiro T, Tenreiro R & Mendes-Faia A (2008) Revisão: as bactérias do ácido láctico do vinho- Parte I. *Ciência e Técnica Vitivinícola* **23**: 81-96.

Inês A, Tenreiro T, Tenreiro R & Mendes-Faia A (2009) Revisão: As bactérias do ácido lático do vinho- Parte II. *Ciência e Técnica Vitivinícola* **24**: 1-23.

Jansen SC, van Dusseldorp M, Bottema KC & Dubois AEJ (2003) Intolerance to dietary biogenic amines: a review. *Annals of Allergy, Asthma & Immunology* **91**: 233-241.

Jin L, Richards A & Brown DW (1996) Development of a dual target-PCR for detection and characterization of measles virus in clinical specimens. *Molecular and cellular probes* **10**: 191-200.

Jones GV, White MA, Cooper OR & Storchmann K (2005) Climate Change and Global Wine Quality. *Climatic Change* **73**: 319-343.

Kalač P, Křížek M, Pelikánová T, Langová M & Veškrna O (2005) Contents of polyamines in selected foods. *Food Chemistry* **90**: 561-564.

Kampfer P, Avesani V, Janssens M, Charlier J, De Baere T & Vaneechoutte M (2006) Description of *Wautersiella falsenii* gen. nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. *Int J Syst Evol Microbiol* **56**: 2323-2329.

Kan JJ, Wang K & Chen F (2006) Temporal variation and detection limit of an estuarine bacterioplankton community analyzed by denaturing gradient gel electrophoresis (DGGE). *Aquatic Microbial Ecology* **42**: 7-18.

Kanny G, Gerbaux V, Olszewski A, Frémont S, Empereur F, Nabet F, Cabanis J-C & Moneret-Vautrin D-A (2001) No correlation between wine intolerance and histamine content of wine. *Journal of Allergy and Clinical Immunology* **107**: 375-378.

Karovičová J, Kohajdová, Z. (2003) Biogenic Amines in Food. *Chemical Papers* **59**: 70—79

Kennedy J, Marchesi JR & Dobson ADW (2007) Metagenomic approaches to exploit the biotechnological potential of the microbial consortia of marine sponges. *Applied Microbiology and Biotechnology* **75**: 11-20.

Kennedy J, Baker P, Piper C*, et al.* (2009) Isolation and Analysis of Bacteria with Antimicrobial Activities from the Marine Sponge *Haliclona simulans* Collected from Irish Waters. *Marine Biotechnology* **11**: 384-396.

Kim TK, Garson MJ & Fuerst JA (2005) Marine actinomycetes related to the 'Salinospora' group from the Great Barrier Reef sponge Pseudoceratina clavata. *Environmental Microbiology* **7**: 509-518.

Komaki H, Ichikawa N, Hosoyama A, Takahashi-Nakaguchi A, Matsuzawa T, Suzuki K, Fujita N & Gonoi T (2014) Genome based analysis of type-I polyketide synthase and nonribosomal peptide synthetase gene clusters in seven strains of five representative Nocardia species. *BMC Genomics* **15**: 1-11.

König H, Unden G & Fröhlich J (2009) *Biology of Microorganisms on Grapes, in Must and in Wine*. Springer-Verlag Berlin Heidelberg.

Konings WN, Lolkema JS, Bolhuis H, vanVeen HW, Poolman B & Driessen AJM (1997) The role of transport processes in survival of lactic acid bacteria - Energy transduction and multidrug resistance. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **71**: 117-128.

Ladero V, Cañedo E, Pérez M, Martín MC, Fernández M & Alvarez MA (2012) Multiplex qPCR for the detection and quantification of putrescine-producing lactic acid bacteria in dairy products. *Food Control* **27**: 307-313.

Ladero V, Coton M, Fernández M, Buron N, Martín MC, Guichard H, Coton E & Alvarez MA (2011) Biogenic amines content in Spanish and French natural ciders: Application of qPCR for quantitative detection of biogenic amine-producers. *Food Microbiology* **28**: 554-561.

Landegren U, Kaiser R, Caskey CT & Hood L (1988) DNA diagnostics--molecular techniques and automation. *Science (New York, NY)* **242**: 229-237.

Landete JM, Ferrer S & Pardo I (2005) Which lactic acid bacteria are responsible for histamine production in wine? *Journal of Applied Microbiology* **99**: 580-586.

Landete JM, Ferrer S & Pardo I (2007) Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control* **18**: 1569-1574.

Lane DJ, Weisburg WG, Barns SM & Pelletier DA (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Lavizzari T, Breccia M, Bover-Cid S, Vidal-Carou MC & Veciana-Nogués MT (2010) Histamine, Cadaverine, and Putrescine Produced *In Vitro* by *Enterobacteriaceae* and *Pseudomonadaceae* Isolated from Spinach. *Journal of Food Protection* **73**: 385-389.

Le Jeune C, Lonvaud-Funel A, ten Brink B, Hofstra H & van der Vossen JM (1995) Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. *The Journal of applied bacteriology* **78**: 316-326.

Lehane L & Olley J (2000) Histamine fish poisoning revisited. *International Journal of Food Microbiology* **58**: 1-37.

Leitão MC, Marques AP & San Romão MV (2005) A survey of biogenic amines in commercial Portuguese wines. *Food Control* **16**: 199-204.

Leitão MC, Teixeira HC, Barreto Crespo MT & San Romão MV (2000) Biogenic Amines Occurrence in Wine. Amino Acid Decarboxylase and Proteolytic Activities Expression by *Oenococcus oeni*. *Journal of Agricultural and Food Chemistry* **48**: 2780-2784.

Lidstrom ME & Chistoserdova L (2002) Plants in the Pink: Cytokinin Production by *Methylobacterium*. *Journal of Bacteriology* **184**: 1818-1818.

Lonvaud-Funel A (2001) Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters* **199**: 9-13.

Lonvaud-Funel A & Joyeux A (1994) Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. *The Journal of applied bacteriology* **77**: 401-407.

Lucas P & Lonvaud-Funel A (2002) Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. *Fems Microbiology Letters* **211**: 85-89.

Lucas P, Landete J, Coton M, Coton E & Lonvaud-Funel A (2003) The tyrosine decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation in tyramine-producing bacteria. *Fems Microbiology Letters* **229**: 65-71.

Lucas PM, Wolken WAM, Claisse O, Lolkema JS & Lonvaud-Funel A (2005) Histamine-Producing Pathway Encoded on an Unstable Plasmid in *Lactobacillus hilgardii* 0006. *Applied and Environmental Microbiology* **71**: 1417-1424.

Lucas PM, Blancato VS, Claisse O, Magni C, Lolkema JS & Lonvaud-Funel A (2007) Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. *Microbiology* **153**: 2221-2230.

Ma DQ, Yanofsky MF, Gordon MP & Nester EW (1987) Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine tumors in China. *Applied and Environmental Microbiology* **53**: 1338-1343.

Madhaiyan M, Poonguzhali S, Kwon SW & Sa TM (2010) Bacillus methylotrophicus sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *Int J Syst Evol Microbiol* **60**: 2490-2495.

Marcobal Á, de Las Rivas B, Moreno-Arribas MV & Muñoz R (2006) Evidence for Horizontal Gene Transfer as Origin of Putrescine Production in *Oenococcus oeni* RM83. *Applied and Environmental Microbiology* **72**: 7954-7958.

Marques AP, Leitão MC & San Romão MV (2008) Biogenic amines in wines: Influence of oenological factors. *Food Chemistry* **107**: 853-860.

Martin MC, Fernandez M, Linares DM & Alvarez MA (2005) Sequencing, characterization and transcriptional analysis of the histidine decarboxylase operon of *Lactobacillus buchneri*. *Microbiology-Sgm* **151**: 1219-1228.

Martins G, Lauga B Fau - Miot-Sertier C, Miot-Sertier C Fau - Mercier A, Mercier A Fau - Lonvaud A, Lonvaud A Fau - Soulas M-L, Soulas Ml Fau - Soulas G, Soulas G Fau - Masneuf-Pomarede I & Masneuf-Pomarede I (2013) Characterization of epiphytic bacterial communities from grapes, leaves, bark and soil of grapevine plants grown, and their relations.

Martuscelli M, Crudele MA, Gardini F & Suzzi G (2000) Biogenic amine formation and oxidation by Staphylococcus xylosus strains from artisanal fermented sausages. *Lett Appl Microbiol* **31**: 228-232.

Miller KI, Qing C, Sze DM-Y, Roufogalis BD & Neilan BA (2012) Culturable Endophytes of Medicinal Plants and the Genetic Basis for Their Bioactivity. *Microbial Ecology* **64**: 431-449.

Min J-S, Lee S-O, Jang A, Lee M & Kim Y (2004) Production of Biogenic Amines by Microflora Inoculated in Meats. *Asian Australas J Anim Sci* **17**: 1472-1478.

Molenaar D, Bosscher JS, ten Brink B, Driessen AJ & Konings WN (1993) Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in Lactobacillus buchneri. *Journal of Bacteriology* **175**: 2864-2870.

Moreno-Arribas MV, Polo MC, Jorganes F & Muñoz R (2003) Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *International Journal of Food Microbiology* **84**: 117-123.

Moreno-Arribas V & Lonvaud-Funel A (1999) Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. *Fems Microbiology Letters* **180**: 55-60.

Muhling M, Woolven-Allen J, Murrell JC & Joint I (2008) Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME J* **2**: 379-392.

Müller CA, Oberauner-Wappis L, Peyman A, Amos GCA, Wellington EMH & Berg G (2015) Mining for Nonribosomal Peptide Synthetase and Polyketide Synthase Genes Revealed a High Level of Diversity in the Sphagnum Bog Metagenome. *Applied and Environmental Microbiology* **81**: 5064-5072.

Muyzer G, Teske A, Wirsen CO & Jannasch HW (1995) Phylogenetic relationships ofThiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* **164**: 165-172.

Naila A, Flint S, Fletcher G, Bremer P & Meerdink G (2010) Control of Biogenic Amines in Food—Existing and Emerging Approaches. *Journal of Food Science* **75**: R139-R150.

Nannelli F, Claisse O, Gindreau E, De Revel G, Lonvaud-Funel A & Lucas PM (2008) Determination of lactic acid bacteria producing biogenic amines in wine by quantitative PCR methods. *Letters in Applied Microbiology* **47**: 594-599.

Neilan BA, Dittmann E, Rouhiainen L, Bass RA, Schaub V, Sivonen K & Borner T (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. *J Bacteriol* **181**: 4089-4097.

Nisiotou AA, Rantsiou K, Iliopoulos V, Cocolin L & Nychas GJ (2011) Bacterial species associated with sound and *Botrytis*-infected grapes from a Greek vineyard. *Int J Food Microbiol* **145**: 432-436.

Pachlová V, Buňka F, Buňková L, Purkrtová S, Havlíková Š & Němečková I (2016) Biogenic amines and their producers in Akawi white cheese. *International Journal of Dairy Technology* **69**: 386-392.

Padgett M & Morrison JC (1990) Changes in Grape Berry Exudates during Fruit Development and Their Effect on Mycelial Growth of *Botrytis cinerea*. *Journal of the American Society for Horticultural Science* **115**: 269-273.

Palomo S, González I, de la Cruz M, Martín J, Tormo JR, Anderson M, Hill RT, Vicente F, Reyes F & Genilloud O (2013) Sponge-Derived *Kocuria* and *Micrococcus spp*. as Sources of the New Thiazolyl Peptide Antibiotic Kocurin. *Marine Drugs* **11**: 1071-1086.

Pathom-Aree W, Nogi Y, Ward AC, Horikoshi K, Bull AT & Goodfellow M (2006) *Dermacoccus barathri* sp. nov. and *Dermacoccus profundi* sp. nov., novel actinomycetes isolated from deep-sea mud of the Mariana Trench. *Int J Syst Evol Microbiol* **56**: 2303-2307.

Pinto C & Gomes AC (2016) *Vitis vinifera* microbiome: from basic research to technological development. *BioControl* **61**: 243-256.

Pinto C, Pinho D, Sousa S, Pinheiro M, Egas C & C. Gomes A (2014) Unravelling the Diversity of Grapevine Microbiome. *PLoS ONE* **9**: e85622.

Pollet T, Tadonléké RD & Humbert JF (2011) Spatiotemporal Changes in the Structure and Composition of a Less-Abundant Bacterial Phylum (Planctomycetes) in Two Perialpine Lakes. *Applied and Environmental Microbiology* **77**: 4811-4821.

Pons-Sanchez-Cascado S, Veciana-Nogues MT, Bover-Cid S, Marine-Font A & Vidal-Carou MC (2005) Volatile and biogenic amines, microbiological counts, and bacterial amino acid decarboxylase activity throughout the salt-ripening process of anchovies (Engraulis encrasicholus). *J Food Prot* **68**: 1683-1689.

Prakitchaiwattana CJ, Fleet GH & Heard GM (2004) Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research* **4**: 865-877.

Randazzo CL, Vaughan EE & Caggia C (2006) Artisanal and experimental Pecorino Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR–DGGE analyses. *International Journal of Food Microbiology* **109**: 1-8.

Renouf V, Claisse O & Lonvaud-Funel A (2005) Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Australian Journal of Grape and Wine Research* **11**: 316-327.

Renouf V, Claisse O & Lonvaud-Funel A (2007) Inventory and monitoring of wine microbial consortia. *Applied Microbiology and Biotechnology* **75**: 149-164.

Rice SL, Eitenmiller RR & Koehler PE (1976) Biologically-active amines in food - review. *Journal of Milk and Food Technology* **39**: 353-358.

Riemann L & Middelboe M (2002) Stability of bacterial and viral community compositions in Danish coastal waters as depicted by DNA fingerprinting techniques. *Aquatic Microbial Ecology* **27**: 219-232.

Riemann L, F. Steward G, Fandino LB, Campbell L, Landry MR & Azam F (1999) Bacterial community composition during two consecutive NE Monsoon periods in the Arabian Sea studied by denaturing gradient gel electrophoresis (DGGE) of rRNA genes. *Deep Sea Research Part II: Topical Studies in Oceanography* **46**: 1791-1811.

Rithidech KN, Dunn JJ & Gordon CR (1997) Combining multiplex and touchdown PCR to screen murine microsatellite polymorphisms. *BioTechniques* **23**: 36, 40, 42, 44.

Roberts MS, Nakamura LK & Cohan FM (1996) Bacillus vallismortis sp. nov., a close relative of Bacillus subtilis, isolated from soil in Death Valley, California. *International journal of systematic bacteriology* **46**: 470-475.

Romano A, Trip H, Lonvaud-Funel A, Lolkema JS & Lucas PM (2012) Evidence of Two Functionally Distinct Ornithine Decarboxylation Systems in Lactic Acid Bacteria. *Applied and Environmental Microbiology* **78**: 1953-1961.

Santos MHS (1996) Biogenic amines: their importance in foods. *International Journal of Food Microbiology* **29**: 213-231.

Schelp E, Worley S, Monzingo AF, Ernst S & Robertus JD (2001) pH-induced structural changes regulate histidine decarboxylase activity in *Lactobacillus* 30a1. *Journal of Molecular Biology* **306**: 727-732.

Scheublin TR & Leveau JH (2013) Isolation of *Arthrobacter* species from the phyllosphere and demonstration of their epiphytic fitness. *MicrobiologyOpen* **2**: 205-213.

Schiller D, Kruse D, Kneifel H, Krämer R & Burkovski A (2000) Polyamine Transport and Role of *potE* in Response to Osmotic Stress in *Escherichia coli*. *Journal of Bacteriology* **182**: 6247-6249.

Shalaby AR (1996) Significance of biogenic amines to food safety and human health. *Food Research International* **29**: 675-690.

Shannon CE (1948) A Mathematical Theory of Communication. *Bell System Technical Journal* **27**: 379-423.

Sherlock J, Cirigliano V, Petrou M, Tutschek B & Adinolfi M (1998) Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. *Annals of human genetics* **62**: 9-23.

Shuber AP, Skoletsky J, Stern R & Handelin BL (1993) Efficient 12-mutation testing in the CFTR gene: a general model for complex mutation analysis. *Human molecular genetics* **2**: 153-158.

Singh R, Vijjaya & Kabra M (2006) Multiplex PCR for rapid detection of exonal deletions in patients of duchenne muscular dystrophy. *Indian journal of clinical biochemistry : IJCB* **21**: 147-151.

Smit AY, du Toit, W.J.,. du Toit, M (2008) Biogenic Amines in Wine: Understanding the Headache. *South Africa Journal of Enology and Viticulture, Vol 29, No 2, 2008* **29**: 109-126.

Smith TA (1981) Amines in food. *Food Chemistry* **6**: 169-200.

Soufleros EH, Bouloumpasi, E., Zotou, A., Loukou, Z. (2007) Determination of biogenic amines in Greek wines by HPLC and ultraviolet detection after dansylation and examination of factors affecting their presence and concentration. *Food Chemistry* **101** 704-716

Spano G, Russo P, Lonvaud-Funel A*, et al.* (2010) Biogenic amines in fermented foods. *Eur J Clin Nutr* **64**: S95-S100.

Stratton JE, Hutkins RW & Taylor SL (1991) Biogenic-Amines in cheese and other fermented foods - a review. *Journal of Food Protection* **54**: 460-470.

Straub BW, Kicherer M, Schilcher SM & Hammes WP (1995) The formation of biogenic amines by fermentation organisms. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **201**: 79-82.

Sturgill G & Rather PN (2004) Evidence that putrescine acts as an extracellular signal required for swarming in *Proteus mirabilis*. *Molecular Microbiology* **51**: 437-446.

Szegedi E, Bottka, S., Mikulás, J. ,Otten, L. and Sule , S. (2005) Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine. *Vitis* **44**: 49–54

Tabor H & Tabor CW (1972) Biosynthesis and metabolism of 1,4-diaminobutane, spermidine, spermine, and related amines. *Advances in enzymology and related areas of molecular biology* **36**: 203-268.

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.

Taylor GR & Robinson P (1998) The polymerase chain reaction: from functional genomics to high-school practical classes. *Current opinion in biotechnology* **9**: 35-42.

Taylor SL, Guthertz LS, Tillman MLF & Lieber ER (1978) Histamine production by food-borne bacterial species. *Journal of Food Safety* **1**: 173-187.

ten Brink B, Damink C, Joosten HMLJ & Huis in 't Veld JHJ (1990) Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology* **11**: 73-84.

Timmusk S, Grantcharova N & Wagner EGH (2005) *Paenibacillus polymyxa* Invades Plant Roots and Forms Biofilms. *Applied and Environmental Microbiology* **71**: 7292-7300.

Torrea D & Ancín C (2002) Content of Biogenic Amines in a Chardonnay Wine Obtained through Spontaneous and Inoculated Fermentations. *Journal of Agricultural and Food Chemistry* **50**: 4895-4899.

van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD & Maguin E (2002) Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* **82**: 187-216.

Wagar EA (1996) Direct hybridization and amplification applications for the diagnosis of infectious diseases. *Journal of clinical laboratory analysis* **10**: 312-325.

Wang H, Fewer DP, Holm L, Rouhiainen L & Sivonen K (2014) Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 9259-9264.

Wolcott MJ (1992) Advances in nucleic acid-based detection methods. *Clinical Microbiology Reviews* **5**: 370-386.

Wunderlichová L, Buňková L, Koutný M, Jančová P & Buňka F (2014) Formation, Degradation, and Detoxification of Putrescine by Foodborne Bacteria: A Review. *Comprehensive Reviews in Food Science and Food Safety* **13**: 1012-1030.

Zarraonaindia I, Owens SM, Weisenhorn P*, et al.* (2015) The Soil Microbiome Influences Grapevine-Associated Microbiota. *mBio* **6**.

Zimmermann K, Schogl D, Plaimauer B & Mannhalter JW (1996) Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. *BioTechniques* **21**: 480-484.

Zoetendal EG, Akkermans, A. D. L., Akkermans-van Vliet Wilma, M.J., Arjan, G. M. de Visser, W. M. de Vos (2001) The Host Genotype Affects the Bacterial Community in the Human Gastronintestinal Tract. *Microbial Ecology in Health and Disease* **13**: 129-134.

Zou S, Stansfield C & Bridge J (1998) Identification of New Influenza B Virus Variants by Multiplex Reverse Transcription-PCR and the Heteroduplex Mobility Assay. *Journal of Clinical Microbiology* **36**: 1544-1548.

Webpage 1, 07/09/2016, **National Center for Biotechnology Information**, https://www.ncbi.nlm.nih.gov/nuccore/GU369972.1

8. Annexes

Annex I

Table I.i – Results of the Blast in NCBI database, performed with the obtained sequences of the 16S rRNA of the isolates. 11

Isolate	NCBI closest Hit						
	GenBank Acession Number	Description	Query cover	E. Value	Identity	Affiliation	
D(1)_2	KM187280.1	*Curtobacterium sp.* CC5L 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(1)_4	KP279888.1	*Curtobacterium sp.* DP122B 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_7U	KJ744019.1	*Dermacoccus sp*. 100S2a 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Dermacoccaceae*
A(2)_50Z	JQ977481.1	*Dermacoccus sp.* Bma12 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Actinobacteria*	*Dermacoccaceae*
A(2)_45Z	AB211032.1	*Dietzia maris* gene for 16S rRNA, partial sequence, strain: SSCS4	100%	0.0	99%	*Actinobacteria*	*Dietziaceae*
A(2)_39Z	KU601225.1	*Dietzia maris* strain Y1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Dietziaceae*
A(2)_27	JX949725.1	*Agrococcus sp.* MDT2-5 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_32Z	KX289379.1	*Leucobacter sp.* DS31 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_29	KR906508.1	*Leucobacter sp.* HBUM179329 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_9X	KR906508.1	*Leucobacter sp*. HBUM179329 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*

A(2)_30Z	NR_041332.1	*Microbacterium aoyamense* strain KV-492 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_9U	KC430861.1	*Microbacterium esteraromaticum* strain BA1109 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_44.1Z	KX390640.1	*Microbacterium sp.* H83 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_8Z	FJ267583.1	*Microbacterium sp.* I_GA_A_1_16 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Microbacteriaceae*
A(2)_17U	JX949317.1	*Arthrobacter sp.* Hh21 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Micrococcaceae*
A(2)_15U	HQ419278.1	*Arthrobacter sp.* JSM 101049 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Micrococcaceae*
A(2)_8U	HQ419278.1	*Arthrobacter sp*. JSM 101049 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Micrococcaceae*
A(2)_16	KX055834.1	*Kocuria kristinae* strain RUTW4-5 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Actinobacteria*	*Micrococcaceae*
A(2)_20	KX055834.1	*Kocuria kristinae* strain RUTW4-5 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Actinobacteria*	*Micrococcaceae*
A(2)_6X	KP843722.1	*Rhodococcus equi* strain DiscAct4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Nocardiaceae*
A(2)_33Z	KT951673.1	*Rhodococcus sp*. PY11 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Actinobacteria*	*Nocardiaceae*
A(2)_26	KF740325.1	*Roseomonas sp.* CMS4Y-2-2 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Alphaproteobacteria*	*Acetobacteraceae*
D(2)_4	LK391673.1	*Brevundimonas diminuta* partial 16S rRNA gene, isolate KSW 68	100%	0.0	99%	*Alphaproteobacteria*	*Caulobacteraceae*
A(2)_13U	JX908719.1	*Brevundimonas sp*. P10 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Alphaproteobacteria*	*Caulobacteraceae*
D(2)_1.1	KP152637.1	*Brevundimonas sp*. ZQM-218 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Alphaproteobacteria*	*Caulobacteraceae*

D(2)_11	KP152637.1	*Brevundimonas sp.* ZQM-218 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Alphaproteobacteria*	*Caulobacteraceae*
D(2)_8	KP152637.1	*Brevundimonas sp*. ZQM-218 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Alphaproteobacteria*	*Caulobacteraceae*
A(2)_29Z	AB698709.1	*Methylobacterium adhaesivum* gene for 16S ribosomal RNA, partial sequence, strain: 34b	99%	0.0	100%	*Alphaproteobacteria*	*Methylobacteriaceae*
A(2)_49Z	AB698709.1	*Methylobacterium adhaesivum* gene for 16S ribosomal RNA, partial sequence, strain: 34b	100%	0.0	99%	*Alphaproteobacteria*	*Methylobacteriaceae*
A(2)_38Z	KR085941.1	*Methylobacterium tardum* strain IHBB 11162 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Alphaproteobacteria*	*Methylobacteriaceae*
A(2)_47Z	KX150814.1	*Agrobacterium tumefaciens* strain HaTc13 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Alphaproteobacteria*	*Rhizobiaceae*
A(2)_43Z	KF709118.1	*Agrobacterium tumefaciens* strain SM14 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Alphaproteobacteria*	*Rhizobiaceae*
A(2)_12U	AM238684.1	*Wautersiella falsenii* genomovar 1 partial 16S rRNA gene, strain NF 289	99%	0.0	99%	*Bacteroidetes*	*Flavobacteriaceae*
A(2)_23S	KX832639.1	*Bacillus amyloliquefaciens* strain MD81 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_3X	KT381096.1	*Bacillus amyloliquefaciens* strain ZSY-1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_17Z	KU605229.1	*Bacillus anthracis* strain IHB B 15639 16S ribosomal RNA gene, partial sequence	97%	0.0	83%	*Firmicutes*	*Bacillaceae*
A(2)_2X	KT026101.1	*Bacillus cereus* 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_7	KP717557.1	*Bacillus cereus* strain BNi22 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_5.1	KT719454.1	*Bacillus cereus* strain MER_TA_49 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_5Z	KU254657.1	*Bacillus drentensis* strain NA-10 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	*Firmicutes*	*Bacillaceae*

A(1)_14	KX218317.1	*Bacillus megaterium* strain 90 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_22Z	KU605237.1	*Bacillus megaterium* strain IHB B 15710 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_15.1S	KU179346.1	*Bacillus megaterium* strain L43 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_15.1Z	KU179346.1	*Bacillus megaterium* strain L43 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_7Z	KT986109.1	*Bacillus megaterium* strain Lmb044 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_17.2S	KT719412.1	*Bacillus methylotrophicus* strain MER_TA_3.1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(1)_11	KX218301.1	*Bacillus pumilus* strain 86 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_11	KF933629.1	*Bacillus pumilus* strain BDH8 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(1)_9	KX673640.1	*Bacillus pumilus* strain SSRCI03 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_27Z	KX809651.1	*Bacillus safensis* strain ADU20 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_5	KX289483.1	*Bacillus sp*. CSC12(2016) 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_13Z	KR077861.1	*Bacillus sp*. FJAT-25772 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(1)_3	AY289498.1	*Bacillus sp*. IDA4740 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_15Z	AB733565.1	*Bacillus sp.* MBEP11 gene for 16S rRNA, partial sequence	99%	0.0	98%	*Firmicutes*	*Bacillaceae*
A(1)_5	KF740319.1	*Bacillus sp.* MK6Y-6-2 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*

A(2)_10	KX816425.1	*Bacillus sp.* strain 22-12 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_1U	KX816425.1	*Bacillus sp*. strain 22-12 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_5U	KX816425.1	*Bacillus sp.* strain 22-12 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_23Z	KX816443.1	*Bacillus sp.* strain 26-11 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_24Z	KX664466.1	*Bacillus sp*. strain 7-2 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_31Z	KX664467.1	*Bacillus sp.* strain 7-3 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_17.1	KX622617.1	*Bacillus sp*. strain BAB-5955 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_3	KX373984.1	*Bacillus sp.* strain NT4 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_5X	KT720060.1	*Bacillus tequilensis* strain PF3i_1.2 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_21	KX444644.1	*Bacillus thuringiensis* strain B18 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_8	KT986127.1	*Bacillus thuringiensis* strain Lmb062 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_13S	HQ992817.1	*Bacillus vallismortis* strain TD3 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_12	KU356161.1	*Bacillus velezensis* strain D-004 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Bacillaceae*
A(2)_1X	GQ280035.1	*Lysinibacillus fusiformis* strain BJ-25 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Bacillaceae*
A(2)_14U	KJ806305.1	*Pediococcus pentosaceus* strain CG35 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Lactobacillaceae*

A(2)_7X	AB682293.1	*Paenibacillus graminis* gene for 16S rRNA, partial sequence, strain: NBRC 105756	100%	0.0	99%	*Firmicutes*	*Paenibacillaceae*
A(2)_8X	AB682293.1	*Paenibacillus graminis* gene for 16S rRNA, partial sequence, strain: NBRC 105756	100%	0.0	99%	*Firmicutes*	*Paenibacillaceae*
A(2)_28	AY359623.1	*Paenibacillus polymyxa* strain GBR-465 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Paenibacillaceae*
A(2)_30	GU369972.1	*Paenibacillus polymyxa* strain RCP6 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Paenibacillaceae*
A(2)_25	EU071625.1	*Staphylococcus hominis* strain EHFS2_AC2Hb 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Staphylococcaceae*
A(2)_26Z	KT989529.1	*Staphylococcus saprophyticus* subsp. bovis strain E7-5c 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Staphylococcaceae*
A(2)_10U	JX944828.1	*Staphylococcus sp.* Y73 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	*Firmicutes*	*Staphylococcaceae*
A(2)_17	KX349994.1	*Staphylococcus warneri* strain F2-1-6 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Firmicutes*	*Staphylococcaceae*
A(2)_15	KC764978.1	*Enterobacter cloacae* strain T137 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Gammaproteobacteria*	*Enterobacteriaceae*
A(1)_8	KJ127203.1	*Acinetobacter calcoaceticus* strain TF1-9 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	*Gammaproteobacteria*	*Pseudomonadales*

Annex II

Table II.i – Presence of the studied genes in each isolate, linked with their taxonomy.12

Isolate	Affiliation		Closest strain (NCBI closest Hit)	BAs related genes	PKS-I	NRPS
D(1)_2	*Actinobacteria*	*Microbacteriaceae*	*Curtobacterium sp.* CC5L	-	-	-
A(1)_4	*Actinobacteria*	*Microbacteriaceae*	*Curtobacterium sp.* DP122B	*agdi*	-	-
A(2)_7U	*Actinobacteria*	*Dermacoccaceae*	*Dermacoccus sp*. 100S2a	*agdi*	-	-
A(2)_50Z	*Actinobacteria*	*Dermacoccaceae*	*Dermacoccus sp.* Bma12	*agdi* *odc*	-	-

A(2)_45Z	*Actinobacteria*	*Dietziaceae*	*Dietzia maris*	-	-	-
A(2)_39Z	*Actinobacteria*	*Dietziaceae*	*Dietzia maris* strain Y1	*odc*	-	-
A(2)_27	*Actinobacteria*	*Microbacteriaceae*	*Agrococcus sp.* MDT2-5	-	*PKS-I*	-
A(2)_32Z	*Actinobacteria*	*Microbacteriaceae*	*Leucobacter sp.* DS31	*agdi*	-	-
A(2)_29	*Actinobacteria*	*Microbacteriaceae*	*Leucobacter sp.* HBUM179329	*agdi*	-	-
A(2)_9X	*Actinobacteria*	*Microbacteriaceae*	*Leucobacter sp*. HBUM179329	*hdc* *tyrd*	*PKS-I*	-
A(2)_30Z	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium aoyamense* strain KV-492	*agdi* *tyrd*	*PKS-I*	-
A(2)_9U	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium esteraromaticum* strain BA1109	*odc*	-	-
A(2)_44.1Z	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium sp.* H83	*odc*	-	-
A(2)_8Z	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium sp.* I_GA_A_1_16	*agdi*	*PKS-I*	-
A(2)_17U	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter sp.* Hh21	*odc*	-	-
A(2)_15U	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter sp.* JSM 101049	*hdc* *odc*	-	-
A(2)_8U	*Actinobacteria*	*Micrococcaceae*	*Arthrobacter sp*. JSM 101049	*hdc* *agdi*	-	-
A(2)_16	*Actinobacteria*	*Micrococcaceae*	*Kocuria kristinae* strain RUTW4-5	*agdi*	*PKS-I*	-
A(2)_20	*Actinobacteria*	*Micrococcaceae*	*Kocuria kristinae* strain RUTW4-5	*agdi*	*PKS-I*	-
A(2)_6X	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus equi* strain DiscAct4	*odc* *hdc*	*PKS-I*	*NRPS*
A(2)_33Z	*Actinobacteria*	*Nocardiaceae*	*Rhodococcus sp*. PY11	*odc*	-	-
A(2)_26	*Alphaproteobacteria*	*Acetobacteraceae*	*Roseomonas sp.* CMS4Y-2-2	-	-	-
D(2)_4	*Alphaproteobacteria*	*Caulobacteraceae*	*Brevundimonas diminuta* isolate KSW 68	*tyrd*	-	-
A(2)_13U	*Alphaproteobacteria*	*Caulobacteraceae*	*Brevundimonas sp*. P10	*hdc*	-	-
D(2)_1.1	*Alphaproteobacteria*	*Caulobacteraceae*	*Brevundimonas sp*. ZQM-218	*tyrd*	-	-
D(2)_11	*Alphaproteobacteria*	*Caulobacteraceae*	*Brevundimonas sp.* ZQM-218	*tyrd*	-	-
D(2)_8	*Alphaproteobacteria*	*Caulobacteraceae*	*Brevundimonas sp*. ZQM-218	-	-	-

A(2)_29Z	*Alphaproteobacteria*	*Methylobacteriaceae*	*Methylobacterium adhaesivum* strain: 34b	-	-	-
A(2)_49Z	*Alphaproteobacteria*	*Methylobacteriaceae*	*Methylobacterium adhaesivum* strain: 34b	-	-	-
A(2)_38Z	*Alphaproteobacteria*	*Methylobacteriaceae*	*Methylobacterium tardum* strain IHBB 11162	*agdi*	*PKS-I*	-
A(2)_47Z	*Alphaproteobacteria*	*Rhizobiaceae*	*Agrobacterium tumefaciens* strain HaTc13	*agdi*	-	-
A(2)_43Z	*Alphaproteobacteria*	*Rhizobiaceae*	*Agrobacterium tumefaciens* strain SM14	*odc* *tyrd* *agdi*	-	-
A(2)_12U	*Bacteroidetes*	*Flavobacteriaceae*	*Wautersiella falsenii* genomovar 1 strain NF 289	*hdc*	*PKS-I*	-
A(2)_23S	*Firmicutes*	*Bacillaceae*	*Bacillus amyloliquefaciens* strain MD81	-	-	-
A(2)_3X	*Firmicutes*	*Bacillaceae*	*Bacillus amyloliquefaciens* strain ZSY-1	*agdi* *hdc*	*PKS-I*	*NRPS*
A(2)_17Z	*Firmicutes*	*Bacillaceae*	*Bacillus anthracis* strain IHB B 15639	*odc*	*PKS-I*	-
A(2)_2X	*Firmicutes*	*Bacillaceae*	*Bacillus cereus*	*odc*	-	-
A(2)_7	*Firmicutes*	*Bacillaceae*	*Bacillus cereus* strain BNi22	-	-	-
A(2)_5.1	*Firmicutes*	*Bacillaceae*	*Bacillus cereus* strain MER_TA_49	-	-	-
A(2)_5Z	*Firmicutes*	*Bacillaceae*	*Bacillus drentensis* strain NA-10	*agdi*	-	-
A(1)_14	*Firmicutes*	*Bacillaceae*	*Bacillus megaterium* strain 90	-	-	*NRPS*
A(2)_22Z	*Firmicutes*	*Bacillaceae*	*Bacillus megaterium* strain IHB B 15710	-	-	-
A(2)_15.1S	*Firmicutes*	*Bacillaceae*	*Bacillus megaterium* strain L43	-	-	-
A(2)_15.1Z	*Firmicutes*	*Bacillaceae*	*Bacillus megaterium* strain L43	-	-	-
A(2)_7Z	*Firmicutes*	*Bacillaceae*	*Bacillus megaterium* strain Lmb044	-	-	-
A(2)_17.2S	*Firmicutes*	*Bacillaceae*	*Bacillus methylotrophicus* strain MER_TA_3.1	*odc*	-	-
A(1)_11	*Firmicutes*	*Bacillaceae*	*Bacillus pumilus* strain 86	-	-	*NRPS*
A(2)_11	*Firmicutes*	*Bacillaceae*	*Bacillus pumilus* strain BDH8	*hdc*	-	-
A(1)_9	*Firmicutes*	*Bacillaceae*	*Bacillus pumilus* strain SSRCI03	-	-	*NRPS*
A(2)_27Z	*Firmicutes*	*Bacillaceae*	*Bacillus safensis* strain ADU20	-	-	-

A(2)_5	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. CSC12(2016)	-	-	-
A(2)_13Z	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. FJAT-25772	*agdi*	-	-
A(1)_3	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. IDA4740	*agdi*	-	-
A(2)_15Z	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* MBEP11	*odc*	*PKS-I*	-
A(1)_5	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* MK6Y-6-2	*agdi*	-	-
A(2)_10	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* strain 22-12	-	-	-
A(2)_1U	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. strain 22-12	-	-	-
A(2)_5U	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* strain 22-12	-	-	-
A(2)_23Z	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* strain 26-11	*hdc*	-	-
A(2)_24Z	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. strain 7-2	-	*PKS-I*	-
A(2)_31Z	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* strain 7-3	*agdi*	-	-
A(2)_17.1	*Firmicutes*	*Bacillaceae*	*Bacillus sp*. strain BAB-5955	-	-	-
A(2)_3	*Firmicutes*	*Bacillaceae*	*Bacillus sp.* strain NT4	-	-	-
A(2)_5X	*Firmicutes*	*Bacillaceae*	*Bacillus tequilensis* strain PF3i_1.2	*agdi* *hdc*	*PKS-I*	*NRPS*
A(2)_21	*Firmicutes*	*Bacillaceae*	*Bacillus thuringiensis* strain B18	*tyrd*	-	-
A(2)_8	*Firmicutes*	*Bacillaceae*	*Bacillus thuringiensis* strain Lmb062	-	-	-
A(2)_13S	*Firmicutes*	*Bacillaceae*	*Bacillus vallismortis* strain TD3	*tyrd* *odc*		
A(2)_12	*Firmicutes*	*Bacillaceae*	*Bacillus velezensis* strain D-004	*tyrd*	*PKS-I*	-
A(2)_1X	*Firmicutes*	*Bacillaceae*	*Lysinibacillus fusiformis* strain BJ-25	*agdi* *hdc*	-	-
A(2)_14U	*Firmicutes*	*Lactobacillaceae*	*Pediococcus pentosaceus* strain CG35	*hdc*	-	-
A(2)_7X	*Firmicutes*	*Paenibacillaceae*	*Paenibacillus graminis* strain: NBRC 105756	*tyrd*	*PKS-I*	-
A(2)_8X	*Firmicutes*	*Paenibacillaceae*	*Paenibacillus graminis* strain: NBRC 105756	*odc* *hdc* *tyrd*	*PKS-I*	-

A(2)_28	*Firmicutes*	*Paenibacillaceae*	*Paenibacillus polymyxa* strain GBR-465	-	*PKS-I*	*NRPS*
A(2)_30	*Firmicutes*	*Paenibacillaceae*	*Paenibacillus polymyxa* strain RCP6	-	*PKS-I*	*NRPS*
A(2)_25	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus hominis* strain EHFS2_AC2Hb	-	-	-
A(2)_26Z	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus saprophyticus* subsp. bovis strain E7-5c	*hdc*	-	-
A(2)_10U	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus sp.* Y73	*hdc* *odc*	-	-
A(2)_17	*Firmicutes*	*Staphylococcaceae*	*Staphylococcus warneri* strain F2-1-6	-	-	-
A(2)_15	*Gammaproteobacteria*	*Enterobacteriaceae*	*Enterobacter cloacae* strain T137	*agdi* *ldc*	*PKS-I*	-
A(1)_8	*Gammaproteobacteria*	*Pseudomonadales*	*Acinetobacter calcoaceticus* strain TF1-9	-	-	*NRPS*